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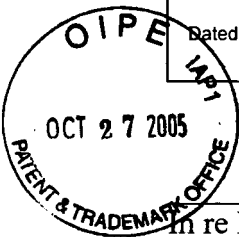
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Dated: October 27, 2005

Signature: _____

(Grace Yu)

Docket No.: 300622001610
(PATENT)



IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Philip J. BARR et al.

Application No.: 09/851,650

Confirmation No.: 4395

Filed: May 8, 2001

Art Unit: 1656

For: PRODUCTION OF POLYKETIDES IN
BACTERIA AND YEAST

Examiner: K. Kerr

APPEAL BRIEF

MS Appeal Brief - Patents
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

As required under § 41.37(a), this brief is filed more than two months after the Notice of Appeal filed in this case on April 27, 2005, and is in furtherance of said Notice of Appeal. The fees required under § 41.20(b)(2) are dealt with in the accompanying Transmittal of Appeal Brief. Additionally, a request for a four-month extension of time is hereby requested.

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I. REAL PARTY IN INTEREST

Kosan Biosciences, Inc. of 3832 Bay Center Place, Hayward, California, 94545, is the real party in interest and sole assignee of the present application by virtue of the assignment recorded at reel/frame number 009419/0446.

II. RELATED APPEALS, INTERFERENCES, AND JUDICIAL PROCEEDINGS

There are no other appeals, interferences, or judicial proceedings which will directly affect or be directly affected by or have a bearing on the Board's decision in this appeal.

III. STATUS OF CLAIMS

Claims 1, 3, 5-6, 8, 10, 12-14, 40, and 41 are pending. Claims 2, 4, 7, 9, 11, 15-39 have been cancelled. Applicants appeal the final rejection of claims 1, 3, 5, 6, 8, 10, 12-14, 40 and 41, in the Office Action mailed January 27, 2005. Claims 1, 3, 5, 6, 8, 10, 12-14 stand rejected under 35 U.S.C. § 112, first paragraph for an alleged lack of written description and for allegedly lacking enablement. Claims 8, 10, 12-14, 40 and 41 stand rejected under 35 U.S.C. § 112, second paragraph, for allegedly being indefinite.

IV. STATUS OF AMENDMENTS

Applicant did not file an Amendment After Final Rejection.

V. SUMMARY OF CLAIMED SUBJECT MATTER

The invention relates, in part, to the production of polyketides in recombinant *E. coli* or yeast host cells. Polyketides are natural products that are assembled by a series of decarboxylation/condensation reactions catalyzed by polyketide synthase (PKS). The grown chain is assembled on an acyl carrier protein (ACP), which requires the attachment of a 4'phosphopantetheine cofactor to produce an active holo-ACP. The present invention embodies the

discovery that recombinant *E. coli* or yeast host cells normally incapable of producing polyketides can be modified to contain an expression systems encoding a polyketide synthase (PKS) and an expression system comprising a holo acyl carrier protein (ACP) synthase which is not associated with fatty acid synthesis, and that pantetheinylates the PKS. By doing so, these modified *E. coli* or yeast host cells become capable of producing polyketides.

VI. GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

The issues on appeal can be separated into two groups, the first group relates to the alleged lack of written description and enablement of the claim language reciting a holo acyl carrier protein (ACP) synthase which is not associated with fatty acid synthesis. The second group relates to the definiteness of the claim language in claims 8, 10, 12-14, 40 and 41.

VII. ARGUMENT

A. The Pending Claims Satisfy the Written Description Requirement

Claims 1, 3, 5, 6, 8, 10, 12-14 and 40 stand rejected under 35 U.S.C. § 112, first paragraph for allegedly lacking sufficient written support. The patent statute requires that an applicant describe a claimed invention in sufficient detail such that one of ordinary skill in the art could reasonably conclude that the applicant was in possession of the claimed invention at the time the application was filed. *Enzo Biochem, Inc. v. Gen-Probe Inc.*, 296 F.3d 1316, 1330 (Fed. Cir. 2002). The claimed invention recites the use of ACP synthases that are not associated with fatty acid synthesis. The Examiner finally rejected the pending claims by alleging that the present application does not sufficiently describe this genus of enzymes. Applicants respectfully disagree.

The present application discloses which ACP synthases are to be used with the claimed invention in sufficient detail as to permit one of ordinary skill in the art reasonably conclude that applicants were in possession of the claimed invention at the time of filing. In relevant part, the

pending claims require the use of ACP synthases that are not associated with fatty acid synthesis.¹ At the time the invention was filed, those of ordinary skill in the relevant art classified ACP synthases also known as phosphopantetheine transferases (EC 2.7.8.7, see, *e.g.*, <http://www.chem.qmul.ac.uk/iubmb/enzyme/>) into three groups according to their sequence homologies and substrate spectrum. The first group is the ACPS type, named for the AcpS of *E. coli*. ACP synthases of this type are about 120 amino acids long and are found in almost all microorganisms. See Mootz, et al. (2001), **J. Biol. Chem.** 276 (40): 37289-37298.) The second group of ACP synthases is modeled after Sfp of *B. subtilis*. These enzymes are typically about 240 amino acids in length. *Id.* The third group of ACPases is an integrated enzyme found in the C-terminal domain of the multifunctional FAS2. *Id.* In light of this classification system which was known in the art at the relevant time period, the specifications use of the language “not associated with fatty acid synthesis” was sufficient to inform one of ordinary skill in the art that ACP synthases of the first group were to be used with the teachings of the specification.

It would have been trivial for one of ordinary skill in the art to distinguish between an ACP synthase that was not associated with fatty acid synthesis and one that was associated at the time the application was filed. At the time the application was filed it was understood by those of ordinary skill in the art that certain ACP synthases were associated with fatty acid production and others that were not associated. This point is illustrated by the work of Gehring, *et al.*, which discusses the generally inability of *E. coli* to produce polyketides using a fatty acid biosynthesis enzyme. Gehring, *et al.* (1996) **Curr. Biol.** 4:17-24. Additionally, Applicants point to the work of Lambalot *et al.*, who were pioneers in the field to characterize the phosphopantetheinyl transferase superfamily. In their 1996 Chemistry & Biology paper, Lambalot *et al.* describes a new family of ACP synthases that catalyze the transfer of the 4'phosphantetheine group to form holo-ACP by comparing various enzyme sequences to the *E. coli* ACP synthases, ACPS, EntD, o195 and the *B. subtilis* protein Sfp. Lambalot, *et al.* (1996) **Chem Biol.** 3(11):923-36. ACPS and EntD are specific for fatty acid synthase and enterobactin synthetase. The *B. subtilis* Sfp is specific for

¹ . “In general, holo ACP synthases associated with fatty acid synthesis are not suitable; rather, synthases associated specifically with polyketide synthesis or with synthesis of nonribosomal proteins are useful in this regard.”

surfactin synthetase. Thus, those of ordinary skill in the art were aware of a variety of different ACP synthases and could identify those that were suitable to pantetheinylate a minimal modular or fungal PKS.

Table 1 of the Lambalot *et al.* paper illustrates this point by providing a list of ACP synthase homologs, the sequences of all but two of which were publicly available at the relevant time period. This list clearly delineates ACP synthases not associated with fatty acid synthesis and those associated with fatty acid synthesis. The ACP synthases that are approximately 225 amino acids in length are primarily associated with polyketide synthesis or non-ribosomal peptide synthesis while those ACP synthases associated with fatty acid synthesis, such as *E. coli* ACPS, are either markedly shorter in length (*E. coli* ACPS 126 amino acids) or significantly longer, such as FAS2 of *S. cerevisiae*, which is 1894 amino acids long. The one aberration in the table is HI0152 of *H. influenzae*, which Lambalot, *et al.* classified as being associated with fatty acid synthesis. This enzyme proves the rule, however as it was subsequently reclassified as being in the polyketide associated class comprising *sfp*, *gsp*, *hetI*, and *acpT*. (See Accession No. P43954 for HI0152.)

The Examiner alleged in the final Office Action that the claim limitation failed to provide sufficient structure in addition to the recited function to satisfy the written description requirement. Applicants disagree. Those of ordinary skill in the art would have been cognizant at the relevant time that the structural similarities, for example the length of the amino acid sequences of each enzyme, was a distinguishing factor for each group of ACP synthetases. In view of the overall amino acid sequence length (approximately 225 amino acids) for group II, one of ordinary skill in the art could readily understand that ACP synthases of group II were to be used in the presently claimed invention. Furthermore, the differences in amino acid sequence length observed between ACP synthases associated with fatty acid synthesis and those associated with polyketide synthesis provide a concrete selection criterion that one of ordinary skill in the art could use with the disclosed assays, to select appropriate synthases for use with the claimed invention.

Thus, in view of the language of the pending claims, the support for the claim language found in the specification and the state of the art at the relevant time period, one of ordinary skill in the art would reasonably conclude that Applicants were in possession of the claimed subject matter at the time the application was filed. As such, Applicants request that the Board overturn the present rejection.

B. The Pending Claims Satisfy the Enablement Requirement

Claims 1, 3, 5, 6, 8, 10, 12-14 and 40 stand rejected under 35 U.S.C. § 112, first paragraph for allegedly not being supported by an enabling disclosure. “To be enabling, the specification of a patent must teach those skilled in the art to make and use the full scope of the claimed invention without ‘undue experimentation’ ... Nothing more than objective enablement is required, and therefore it is irrelevant whether this teaching is provided through broad terminology or illustrative examples.” *In re Wright*, 999 F.2d 1557, 1561 (Fed. Cir. 1993). Enablement “is not precluded even if some experimentation is necessary, although the amount of experimentation needed must not be unduly extensive.” *See Hybritech Inc. v. Monoclonal Antibodies, Inc.*, 802 F.2d 1367 (Fed. Cir. 1986). Moreover, the Federal Circuit has held that enablement of a genus under § 112, ¶ 1 may be accomplished by showing the enablement of a representative number of species within that genus. *Regents of University of California v. Eli Lilly & Co.*, 119 F.3d 1559 (Fed. Cir. 1997). The Office has alleged that the pending claims are not enabled for all holo ACP synthases that pantetheinylate PKSs. Applicants disagree and respectfully submit that the full scope of the present claims is enabled because a representative number of species within the claimed genus have been disclosed.

At the relevant point in time, one of ordinary skill in the art would be able to practice the full scope of the claimed invention without undue experimentation. Specifically, the full scope of the claimed invention was enabled because the skilled artisan could recognize a suitable ACP synthase based on amino acid length. This point is illustrated by Lambalot *et al.* (1996) paper. Table 1 of this paper lists 23 ACP synthases. ACP synthases which are not associated with fatty acid synthesis are those with enzymes having from approximately 209 to 297 amino acids. Those enzymes with less than about 130 residues or those with substantially greater than about 1,500 residues, such as those in the 1,559 to 1,894 amino acid range, are associated with fatty acid synthesis. Thus, based

on just the amino acid sequence lengths of those enzymes, one of ordinary skill in the art would be guided to which enzymes to use in the claimed method. Furthermore, the identification of new ACP synthases which could possibly function in the claimed invention could be achieved by comparing the amino acid sequence of those new enzymes with the known members of the enzyme family, as discussed and practiced in the Lambalot *et al.* (1996) paper. Accordingly, while some experimentation might be required to practice the claimed invention with any ACP synthase, the quantity and nature of that experimentation would not be “undue” given the level of knowledge that existed in the art coupled with the guidance provided in the specification.

Furthermore, the full scope of the pending claims has been enabled because one of ordinary skill in the art would be cognizant of which ACP synthetases could be used with the claimed invention. The working examples of the present application show the use of three different ACP synthases, each obtained from a different species; the *B. subtilis* enzyme Sfp (Exs. 3, 4 and 6), the *E. coli* enzyme EntD (Ex. 6) and the *B. brevis* enzyme Gsp (Ex. 6). The Lambalot *et al.* (1996) paper disclosed 12 different ACP synthase enzymes (EntD, Sfp, Psf-1, Gsp, etc.), which are not associated with fatty acid synthase and can be practiced within the scope of the claimed invention. Comparing the two disclosures, the present application provides working examples with about a third of the enzymes known at the relevant time point. In view of this, Applicants submit that the working examples provided entitle them to claim the genus disclosed in the pending claims because they have illustrated the claimed subject matter with a representative number of species. As such, the pending claims are supported by an enabling disclosure.

In view of this, Applicants submit that one of ordinary skill in the art would not need to engage in undue experimentation to practice the claimed invention. Accordingly, the Board should overturn the present rejection.

C. The Phrase “Different PKS” Recited in Claims 13 and 14 is Definite

The Examiner rejected claims 13 and 14 under 35 U.S.C. § 112, second paragraph, as allegedly being indefinite in the requirement of “different” PKSs. “[T]he claims, read in light of the

specification, [must] reasonably apprise those skilled in the art and are as precise as the subject matter permits. As a matter of law, no court can demand more.” *Hybritech* at 1385.

The language of claims 13 and 14 is clear and meets the requirements of 35 U.S.C. § 112, second paragraph. The meaning of the phrase “wherein said first and second PKS are different” in claim 13 is clear and simply requires is that the first and second PKSs be “different” from one another. The Examiner complained in the final Office Action that the term was not defined in the specification. No special definition beyond that commonly recognized by those of ordinary skill in the art is required as it is clear on its face. Specifically, the “different” means simply, “not the same” and thus the term encompasses any difference. The Examiner has made this issue overly complex in an attempt to force Applicants to narrow the scope of the claim. Applicants have declined this invitation and request that the Board obviate the present rejection.

D. The Phrase “Encoding A Ketoreductase (KR) Activity” is Definite

The Examiner rejected claim 14 under 35 U.S.C. § 112, second paragraph, as allegedly being indefinite in reciting the phrase “encoding a ketoreductase (KR) activity”. The rejected claim no longer recites this language. Furthermore, the Examiner admitted in the Action that Applicants’ amendments had obviated the grounds of the original rejection by eliminating this language from the claim. (Final Office Action, page 5, paragraph 13.) As such, the reasons supporting this rejection have been overcome and so the rejection should have been withdrawn.

Instead of withdrawing the rejection, the Examiner maintained it arguing that “more appropriate language” would be clearer. Applicants disagree. When read in light of the entire claim and the specification, the skilled artisan would understand that the claimed nucleotide sequence encoding the first or second module would comprise additional nucleic acids which encode a protein having a particular activity. One of ordinary skill in the art reading the claim language in conjunction with the specification would readily understand the metes and bounds of the subject matter Applicants regard as their invention. As such, the present rejection should be overturned by the Board.

E. The Conjunctions Recited are Definite

The Examiner rejected claims 8, 10, and 12-14 under 35 U.S.C. § 112, second paragraph, as allegedly being indefinite. The Examiner's confusion regarding the structure of the claim was apparently the basis for this rejection. The question of clarity is properly judged not from the Examiner's point of view but from the viewpoint of one of ordinary skill in the art.

The conjunctions used to link the various elements of the claim would be clear to one of ordinary skill in the art. For example, the plain meaning of the language recited in independent claim 8 provides that either the elements recited in a) or the elements of b) are to be used with the elements of section c) of this claim. Elements a) and b) are alternatives and thus are linked by the conjunction "or". The element c) is to be included with a) or b) and thus it is listed using the conjunction "and". One of ordinary skill in the art would readily be able to ascertain the metes and bounds of the claim by reading it either alone or in conjunction with the specification. As such, Applicants request the Board to overturn the present rejection.

F. Claims 40 and 41

Applicants admit that claims 40 and 41 contain a typographical error in reciting claim 16 for antecedent basis. These should depend from claims 1 and 8 instead of claims 1 and 16. Applicants will attend to this error once the present appeal has been resolved.

VIII. CLAIMS APPENDIX

A copy of the claims involved in the present appeal is attached hereto as Appendix A. As indicated above, the claims in Appendix A do include the amendments filed by Applicant on October 29, 2004.

IX. EVIDENCE APPENDIX

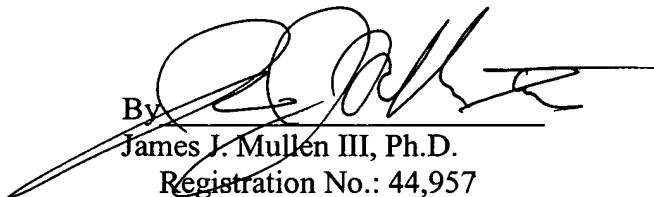
No evidence pursuant to §§ 1.130, 1.131, or 1.132 or entered by or relied upon by the examiner is being submitted. References cited herein are attached hereto as Appendix B.

X. RELATED PROCEEDINGS APPENDIX

No related proceedings are referenced in Heading II above, or copies of decisions in related proceedings are not provided, hence no Appendix is included.

Dated: October 27, 2005

Respectfully submitted,


By _____
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APPENDIX A

Claims Involved in the Appeal of Application Serial No. 09/851,650

1. (previously presented) A modified recombinant *E. coli* or yeast host cell, which, in unmodified form, does not produce polyketides, which cell is modified to contain an expression system that comprises at least one nucleotide sequence that encodes a minimal modular or fungal polyketide synthase (PKS) capable of being expressed and an expression system that comprises at least one nucleotide sequence that encodes a holo acyl carrier protein (ACP) synthase, wherein the ACP synthase pantetheinylates said PKS and said ACP synthase is not associated with fatty acid synthesis,

said minimal PKS comprising a ketosynthase (KS) catalytic region, an acyl transferase (AT) catalytic region, and an ACP domain for a modular PKS or a fungal PKS.

2. (canceled)

3. (previously presented) The modified cell of claim 1 wherein said minimal PKS is the synthase for 6-methyl salicylic acid.

4. (canceled)

5. (original) The modified cell of claim 1 wherein said expression system for said minimal PKS and said expression system for said holo ACP synthase are present on separate vectors.

6. (original) The modified cell of claim 1 wherein at least one of said expression systems is integrated into the host cell chromosome.

7. (canceled)

8. (previously presented) A modified recombinant yeast, *E. coli*, or plant host cell, which, in unmodified form does not produce polyketides, wherein the recombinant host cells is modified to contain either

a) at least a first and a second vector; said first vector containing a first selectable marker and a first expression system and said second vector containing a second selectable marker and a second expression system and optionally additional vectors containing additional selectable markers and expression systems wherein said expression systems contained on said vectors are effective to produce at least a minimal PKS; or

b) at least one vector and a modified chromosome, said one vector containing a first selectable marker and a first expression system and said modified chromosome containing a second expression system and optionally additional vectors containing additional selectable markers and expression systems wherein said expression systems contained on said vectors in combination with said expression system on said chromosome are effective to produce at least a minimal PKS; and

c) a recombinant expression system for a holo ACP synthase capable of being expressed and effective in the pantetheinylation of said PKS;

wherein said minimal PKS comprising a KS catalytic region, an AT catalytic region, and an ACP domain for a modular PKS.

9. (canceled)

10. (previously presented) The cell of claim 8 which further contains an expression system for a cell-based detection system that comprises at least one nucleotide sequence that encodes a protein that is responsive to a polyketide.

11. (canceled)

12. (previously presented) The cell of claim 8 which produces at least a minimal modular PKS and which contains

a) a first vector containing a first selectable marker and a first expression system, wherein said first expression system comprises a nucleotide sequence encoding at least a first module of a PKS operably linked to a promoter operable in said cell; and

b) a second vector containing a second selectable marker and a second expression system, wherein said second expression system comprises a nucleotide sequence encoding at least a second module of a polyketide synthase operably linked to a promoter operable in said cell.

13. (previously presented) The cell of claim 12 wherein said first module is that of a first PKS and said second module is that of a second PKS, wherein said first and second PKS are different.

14. (previously presented) The cell of claim 13 wherein said nucleotide sequence encoding at least a first or a second module further contains a nucleotide sequence encoding a protein having ketoreductase (KR) activity; or

wherein the nucleotide sequence encoding at least a first or a second module further contains a nucleotide sequence encoding a protein having KR activity and a protein having dehydratase (DH) activity; or

wherein said nucleotide sequence encoding at least a first or a second module further contains a nucleotide sequence encoding a protein having KR activity, a protein having DH activity and a protein having enoylreductase (ER) activity; and/or

wherein said nucleotide sequence encoding at least a first or a second module further contains a nucleotide sequence encoding a protein having thioesterase (TE) activity.

15-39. (canceled)

40. (previously presented) The cell of claim 1 or 16 wherein the holo ACP synthase is native to *Bacillus*.

41. (previously presented) The cell of claim 1 or 16 wherein the holo ACP synthase is EntD, GsP, or sfp.

APPENDIX B

Cited References

- EC 2.7.8.7: Description of Phosphopantetheine Transferase Family
- Mootz, et al. (2001), **J. Biol. Chem.** 276 (40): 37289-37298.)
- Gehring, *et al.* (1996) **Curr. Biol.** 4:17-24.
- Lambalot, *et al.* (1996) **Chem Biol.** 3(11):923-36.
- Accession No. P43954 for HI0152.

IUBMB Enzyme Nomenclature

EC 2.7.8.7

Common name: holo-[acyl-carrier-protein] synthase

Reaction: CoA + apo-[acyl-carrier protein] = adenosine 3',5'-bisphosphate + holo-[acyl-carrier protein]

Other names: acyl carrier protein holoprotein (holo-ACP) synthetase; holo-ACP synthetase; coenzyme A:fatty acid synthetase apoenzyme 4'-phosphopantetheine transferase; holosynthase; acyl carrier protein synthetase; holo-ACP synthase

Systematic name: CoA:apo-[acyl-carrier-protein] pantetheinephosphotransferase

Links to other databases: [BRENDA](#), [EXPASY](#), [KEGG](#), [ERGO](#), [PDB](#), CAS registry number: 37278-30-1

References:

1. Elovson, J. and Vagelos, P.R. Acyl carrier protein. X. Acyl carrier protein synthetase. *J. Biol. Chem.* 243 (1968) 3603-3611. [Medline UI: [68313114](#)]
2. Prescott, D.J. and Vagelos, P.R. Acyl carrier protein. *Adv. Enzymol. Relat. Areas Mol. Biol.* 36 (1972) 269-311. [Medline UI: [73004927](#)]

[EC 2.7.8.7 created 1972]

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4'-Phosphopantetheine Transfer in Primary and Secondary Metabolism of *Bacillus subtilis**

Received for publication, April 20, 2001, and in revised form, August 3, 2001
Published, JBC Papers in Press, August 6, 2001, DOI 10.1074/jbc.M103556200

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4'-Phosphopantetheine transferases (PPTases) transfer the 4'-phosphopantetheine moiety of coenzyme A onto a conserved serine residue of acyl carrier proteins (ACPs) of fatty acid and polyketide synthases as well as peptidyl carrier proteins (PCPs) of nonribosomal peptide synthetases. This posttranslational modification converts ACPs and PCPs from their inactive apo into the active holo form. We have investigated the 4'-phosphopantetheinylation reaction in *Bacillus subtilis*, an organism containing in total 43 ACPs and PCPs but only two PPTases, the acyl carrier protein synthase AcpS of primary metabolism and Sfp, a PPTase of secondary metabolism associated with the nonribosomal peptide synthetase for the peptide antibiotic surfactin. We identified and cloned *ycdB* encoding AcpS from *B. subtilis*, which complemented an *Escherichia coli* *acps* disruption mutant. *B. subtilis* AcpS and its substrate ACP were biochemically characterized. AcpS also modified the D-alanyl carrier protein but failed to recognize PCP and an acyl carrier protein of secondary metabolism discovered in this study, designated AcpK, that was not identified by the *Bacillus* genome project. On the other hand, Sfp was able to modify *in vitro* all acyl carrier proteins tested. We thereby extend the reported broad specificity of this enzyme to the homologous ACP. This *in vitro* cross-interaction between primary and secondary metabolism was confirmed under physiological *in vivo* conditions by the construction of a *ycdB* deletion in a *B. subtilis* *sfp*⁺ strain. The genes coding for Sfp and its homolog Gsp from *Bacillus brevis* could also complement the *E. coli* *acps* disruption. These results call into question the essential role of AcpS in strains that contain a Sfp-like PPTase and consequently the suitability of AcpS as a microbial target in such strains.

4'-Phosphopantetheine (Ppant)¹ is an essential prosthetic

group of several acyl carrier proteins involved in pathways of primary and secondary metabolism. These include acyl carrier proteins (ACPs) of fatty acid synthases (FASs), ACPs of polyketide synthases (PKSs), and peptidyl carrier proteins (PCPs) and aryl carrier proteins of nonribosomal peptide synthetases (NRPSs) (1, 2). The free thiol moiety of Ppant serves to covalently bind the acyl reaction intermediates as thioesters during the multistep assembly of the monomeric precursors, typically acetyl, malonyl, and aminoacyl groups. Ppant fulfills two demands in these biosynthetic pathways. First, the intermediates remain covalently tethered to the multifunctional enzyme templates in an energy-rich linkage. Second, the flexibility and length of Ppant (about 20 Å) facilitates the transport of the intermediates to the spatially distinct reaction centers. The Ppant moiety is derived from coenzyme A (CoA) and post-translationally transferred onto an invariant serine side chain. This Mg²⁺-dependent conversion of the apoproteins to the holo proteins is catalyzed by the 4'-phosphopantetheine transferases (PPTases) (1, 2) (see Fig. 1).

Most organisms that employ more than one Ppant-dependent pathway also contain more than one PPTase. For example, *Escherichia coli* has three PPTases, namely the acyl carrier protein synthase AcpS involved in fatty acid synthesis, EntD involved in synthesis of the siderophore enterobactin, and the gene product of *yhhU*, with as yet unknown physiological function (1, 3). Interestingly, PPTases of different pathways can have overlapping selectivity for their cognate acyl carrier protein partner. In *E. coli*, AcpS and EntD have reciprocal specificities, with AcpS only recognizing ACP and EntD only accepting the PCPs of the enterobactin NRPS (1). In contrast, Sfp, the PPTase of the surfactin NRPS of *Bacillus subtilis*, was shown to also phosphopantetheinylate heterologous ACPs of FASs and PKSs (1, 4, 5).

PPTases have been classified in three groups according to their sequence homologies and substrate spectrum. The first group is the AcpS type with AcpS of *Escherichia coli* as the name-giving prototype. PPTases of this type are about 120 aa in length, are found in almost all microorganisms for the modification of fatty acid ACP, and were shown to accept as substrate also ACPs of type II PKS systems (6). The PPTase Sfp of *B. subtilis* is the prototype of the second group. Enzymes of this type are about 240 aa in length and have mostly been found associated with the gene clusters for nonribosomal peptide synthesis. The well characterized Sfp exhibits an extraordinarily broad substrate specificity and could modify all acyl carrier protein substrates tested, including PCPs of NRPS as well as

* Work in the laboratory of M. A. M. was supported by Deutsche Forschungsgemeinschaft and Fonds der Chemischen Industrie. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF260727.

† A Ph.D. fellow of Stiftung Stipendien-Fonds des Verbandes der Chemischen Industrie.

§ These authors contributed equally to this work.

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¹ The abbreviations used are: Ppant, 4'-phosphopantetheine; ACP, acyl carrier protein; AcpK, putative acyl carrier protein localized in the *pksX* cluster; AcpS, acyl carrier protein synthase; CoA, coenzyme A; DCP, D-alanyl carrier protein; FAS, fatty acid synthase; NRPS, nonri-

bosomal peptide synthetase; PCP, peptidyl carrier protein; PCR, polymerase chain reaction; PKS, polyketide synthase; PPTase, 4'-phosphopantetheine transferase; Sfp, PPTase involved in surfactin production; aa, amino acid(s); bp, base pair(s); kb, kilobase pair(s); HPLC, high pressure liquid chromatography; MES, 4-morpholineethanesulfonic acid.

TABLE I
Plasmids used in this study

Plasmid name	Relevant characteristics	Origin or reference
pTZ18R, pTZ19R	ColE1 ori; <i>bla</i> ⁺ ; <i>E. coli</i> cloning vectors	Accession numbers L08956 and L08957
pTrc99a	ColE1 ori; <i>bla</i> ⁺ ; <i>E. coli</i> cloning and expression vector	Accession number U13872
pDR67	ColE1 ori; <i>bla</i> ⁺ ; <i>amyE</i> :: <i>cat</i> ⁺ ; <i>B. subtilis</i> integration vector	Ref. 38
pUC18-nrdD	pMB1 ori; <i>bla</i> ⁺ ; 2994-bp PCR fragment containing <i>nrdD</i> and an upstream fragment	Unpublished, gift from Philippe Marlière
pUC18-nrdD::acps-spc	<i>nrdD</i> :: <i>acps</i> - <i>spc</i> ⁺ ; integration of <i>acps</i> in the <i>nrdD</i> locus of <i>E. coli</i>	This work
pTrc99a-5'-acps::kan-3'	<i>acps</i> :: <i>kan</i> ⁺ ; disruption plasmid for <i>acps</i> <i>E. coli</i>	This work
pTZ18-5'- <i>AydcB</i> :: <i>spc</i> -3'	<i>AydcB</i> :: <i>spc</i> ⁺ ; deletion plasmid for <i>ycdB</i> <i>B. subtilis</i>	This work
pDR67- <i>ycdB</i>	<i>amyE</i> :: <i>ycdB</i> - <i>cat</i> ; integration plasmid for <i>ycdB</i> <i>B. subtilis</i>	This work
pTZ18- <i>ycdB</i>	Expression plasmid for AcpS <i>B. subtilis</i>	This work
pQE60-acpK	Expression plasmid for AcpK-His ₆	This work
pQE60-acpA	Expression plasmid for ACP-His ₆ (<i>B. subtilis</i>)	This work
pQE60-dltC	Expression plasmid for DCP-His ₆ (<i>B. subtilis</i>)	This work
pUC8-sfp	Expression plasmid for Sfp	This work
pTZ19-gsp	Expression plasmid for Gsp	Ref. 33 This work

Construction of the Disruption Plasmid pTZ18R-5'-acps::kan-3' for *E. coli*—Using oligonucleotides E3 (5'-ATATCTAGACCATGACGTATCGTTATC-3') and E4 (5'-ATACCATGGTTCTACTCTGGAAGTAGAG-3'), a 2080-kb fragment was amplified from chromosomal DNA of *E. coli* K-12 that comprised 990 bp upstream and 692 bp downstream of *acps*. This fragment was cloned into pTrc99a using the *Nco*I and *Xba*I sites introduced with the oligonucleotides. The resulting plasmid pTrc99a-5'-acps-3' was then linearized at the single *Sac*I site, which is localized at position 251 of the 381-bp *acps* gene. The kanamycin resistance cassette (*kan*^r) was excised from vector pDG782 (16) with *Eco*RI and *Bgl*II and cloned into pTZ18R to give pTZ18R-kan. The *kan* cassette was then amplified by PCR with oligonucleotides 5'-ATAGAGCTCGACTCACTATAGGGAATTC-3' and 5'-ATAGAGCTCTAAACGACGGCCAGTG-3' from pTZ18R-kan, cut with *Sac*I and ligated with the linearized fragment of pTZ18R-5'-acps-3' to give pTrc99a-5'-acps::kan-3'.

Construction of pTZ18R-*ycdB*-*ycdB* was amplified by PCR from chromosomal DNA of *B. subtilis* JH642 using oligonucleotides 5'-ATAAGCTTCATTTAAATAGTACGTACGC-3' and 5'-TATAAGATCTC-ACTATCAAATATATGAGTGG-3' and cloned blunt-ended into pTZ18R that was linearized with *Hinc*II. The right orientation of *ycdB* under control of the *lac* promoter of pTZ18R-*ycdB* was verified by restriction analysis and sequencing.

Construction of pDR67-*ycdB*—The *acps* gene of *B. subtilis* was cut out of pTZ18R-*ycdB* with *Hind*III and *Bgl*II and cloned between the *Hind*III and *Bgl*II sites of pDR67 to give pDR67-*ycdB*. pDR67 lacks an origin of replication for *B. subtilis* but can integrate into the *amyE* locus of the genome via the *amyE* front and *amyE* back fragments upstream and downstream of the multiple cloning site of the plasmid. The *cat*^r cassette conferring chloramphenicol resistance, which is also located between the two *amyE* fragments, serves to select for the integration. Inserts cloned into the multiple cloning site are under the control of the weak and isopropyl- β -D-thiogalactopyranoside-inducible *spac* promoter, which is leaky in rich media according to our experience.

Construction of the Disruption Plasmid for *ycdB* of *B. subtilis* pTZ18R-5'-*AydcB*::*spc*-3'—To clone flanking regions of *ycdB*, a fragment from 992 bp upstream to 994 bp downstream of *ycdB* was amplified from chromosomal DNA of *B. subtilis* MR168 by PCR with oligonucleotides 5'-ATAGGATCCAGCCTTCATTTTAAAGTGG-3' (primer 1 in Fig. 6) and 5'-AATCTGCAGCAATCTGGGCTTTTCCTG-3' (primer 2 in Fig. 6) and cloned into the *Bam*HI and *Pst*I sites of pTZ18R. The resulting plasmid pTZ18-5'-*ycdB*-3' then served as a template for an inverse PCR with oligonucleotides 5'-ATAGATATCATGTATGATAACCTCC-3' and 5'-ATAGATATCTAGTCTGCATATTAGGG-3' (introducing *Eco*RV restriction sites) to replace the entire *ycdB* with the *spc*⁺ cassette of pDG1726 (16), which was excised with *Eco*RV and *Hinc*II, to give pTZ18R-5'-*AydcB*::*spc*-3'.

Construction of pTZ19-gsp—The *Hind*III-*Pst*I fragment containing the *gsp* gene was excised from pGsp+ (17) and ligated into pTZ19R to give pTZ19-gsp.

Construction of pQE60-ACP—The *acpA* gene encoding ACP was PCR-amplified with oligonucleotides 5'-AATCCATGGCAGACATATAGAGCGT-3' and 5'-TTTGGATCCTTGCTGGTTTGTATGTAGTTT-CAC-3' from chromosomal DNA of *B. subtilis* MR168 and ligated into

the *Nco*I and *Bam*HI sites of pQE60 to give the expression plasmid pQE60-acpA. The encoded recombinant protein carries the C-terminal tag GSRSHHHHHH.

Construction of pQE60-acpK—The *acpK* gene was amplified by PCR with oligonucleotides 5'-TATCCATGGATAAACAGAGAACTTTTG-3' and 5'-TATAGATCTGGCAGATTGCACCTTTGTC-3' from chromosomal DNA of *B. subtilis* MR168. The amplified fragment was digested with *Nco*I and *Bgl*II and ligated into the *Nco*I and *Bam*HI sites of pQE60 to create the expression plasmid pQE60-acpK encoding the recombinant AcpK with a C-terminal tag GSGSHHHHHH.

Construction of pQE60-dltC—The *dltC* gene encoding the D-alanyl carrier protein (DCP) was amplified by PCR with oligonucleotides 5'-ATACCATGGATTTTAAACAAGAGG-3' and 5'-ATAAGATCTTTTCACTCAGACAGCT-3' from chromosomal DNA of *B. subtilis* MR168 and ligated into the *Nco*I and *Bgl*II sites of pQE60. The resulting plasmid pQE60-dltC encodes the recombinant DCP with a C-terminal tag RSHHHHHH.

Overproduction and Purification of Recombinant Proteins—*E. coli* M15/pREP4 was transformed with pTZ18-*ycdB* to give strain RF3. 5 ml of an overnight culture of RF3 in LB were used to inoculate 500 ml of the same medium. Cells were grown at 37 °C and 300 rpm until an *A*₆₀₀ of 0.7 was reached. The culture was then induced with 0.25 mM isopropyl- β -D-thiogalactopyranoside and grown at 37 °C and 300 rpm for 3 h. Cells were harvested by centrifugation at 4,500 \times *g* at 4 °C, resuspended in 50 mM Tris/HCl (pH 7.8), and disrupted by three passages through a cooled French pressure cell. The resulting cell extract was centrifuged at 36,000 \times *g* at 4 °C for 30 min. Ammonium sulfate was added to the supernatant to a final concentration of 25% saturation. The resulting protein suspension was stirred at 4 °C for 1 h and centrifuged at 36,000 \times *g* and 4 °C for 45 min. Subsequently, the pellet was discarded, and ammonium sulfate was added to the supernatant to a final concentration of 40% saturation. The suspension was stirred again at 4 °C for 1 h and centrifuged as described above. The resulting pellet was resuspended in 50 mM Tris/HCl, 1 M (NH₄)₂SO₄ (HIC buffer A, pH 7.8) and centrifuged as described above, and the supernatant was applied to a High-Load™ 26/10 phenyl-Sepharose column (Amersham Pharmacia Biotech) that had been equilibrated with HIC buffer A. The column was washed with buffer A at a flow rate of 1 ml/min, and the protein was eluted with a linear gradient of 1.0 to 0 M (NH₄)₂SO₄ in HIC buffer A; 4-ml fractions were collected. The presence of AcpS in the fractions was detected by SDS-polyacrylamide gel electrophoresis analysis (15% Laemmli gels). Fractions containing AcpS were pooled and concentrated by 40% (NH₄)₂SO₄ precipitation as described above. The resulting pellet was resuspended in 50 mM NaH₂PO₄/NaHPO₄ (pH 7.0), centrifuged as described above, and applied to a Superdex™ G75 26/60 gel filtration column (Amersham Pharmacia Biotech) that had been equilibrated with 50 mM NaH₂PO₄/NaHPO₄ (pH 7.0); 4-ml fractions were collected. Fractions containing AcpS were collected, pooled, and applied to a 6-ml Resource™ 15 S column (Amersham Pharmacia Biotech) that had been equilibrated with 50 mM NaH₂PO₄/NaHPO₄ (CAT buffer A, pH 7.0). The column was washed with CAT buffer A, and the protein was eluted with a linear gradient of 0–0.5 M NaCl in CAT buffer A while collecting 2-ml fractions. The AcpS-containing fractions

were collected and applied to a Superdex™ G75 26/60 gel filtration column that had been equilibrated with 50 mM Tris/HCl, 2 mM dithiothreitol (assay buffer, pH 8.8). Fractions containing AcpS were collected, analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, adjusted with glycerol to a final concentration of 10% (v/v), and stored in small aliquots at -80°C .

E. coli M15/pREP4 was transformed with pQE60-acpA, pQE60-acpK, and pQE60-dltC to give strains RF1, HM403, and RF4, respectively, for the production of the His₆ fusion proteins ACP, AcpK, and DCP. Cells were grown, induced, harvested, and disrupted, and the crude cell extract was centrifuged as described above for RF3. Protein purification using Ni²⁺ affinity chromatography was carried out as previously described (18). Purified proteins were dialyzed against assay buffer, brought to 10% glycerol (v/v), and stored at -80°C . TycC3-PCP, hereafter referred to as PCP, and Sfp-His₆ were produced and purified as previously described (8, 19). Protein concentrations were determined based on the calculated extinction coefficient at 280 nm: AcpS, $6,520\text{ M}^{-1}\text{ cm}^{-1}$; ACP-His₆, $1,280\text{ M}^{-1}\text{ cm}^{-1}$; DCP-His₆, $5,810\text{ M}^{-1}\text{ cm}^{-1}$; AcpK-His₆, $1,400\text{ M}^{-1}\text{ cm}^{-1}$.

Biochemical Assays with *B. subtilis* AcpS and Sfp—AcpS and Sfp activity was assayed by using a radioactive assay method essentially as described previously (15). This method measures the incorporation of the ³H-labeled 4'-phosphopantetheine group from [³H]CoA into apo-ACP or other acyl carrier proteins. Reaction mixtures containing 50 mM Tris/HCl, pH 8.8, 12.5 mM MgCl₂, 2 mM dithiothreitol, a 6–100 μM concentration of the respective acyl carrier protein, 2–20 μM CoA, 119–475 nM [³H]CoA (specific activity: 40 Ci/mmol, 0.95 mCi/ml), 2.2 μM to 5.6 nM AcpS of *B. subtilis*, or 0.8 μM Sfp were incubated at 37°C for 5–30 min. Reactions were stopped by the addition of 0.8 ml of ice-cold trichloroacetic acid (10%) and 15 μl of bovine serum albumin (25 mg/ml). Precipitated protein was collected by centrifugation at 13,000 rpm and 4°C for 15 min. The pellet was washed twice with 0.8 ml of ice-cold trichloroacetic acid and resuspended in 200 μl of formic acid. The resulting suspension was mixed with 3.5 ml of Rotiszint Eco Plus scintillation fluid (Roth) and counted using a 1900CA Tri-Carb liquid scintillation analyzer (Packard).

For kinetic studies, reaction mixtures contained, unless otherwise indicated, 50 mM Tris/HCl, pH 8.8 (75 mM MES, pH 6.0, in the case of Sfp), 12.5 mM MgCl₂, 2 mM dithiothreitol, 2–200 μM apo-ACP of *B. subtilis* (apo/olo mixture) or 2–60 μM AcpK, 2–1000 μM CoA, and 5.6 nM AcpS of *B. subtilis* or 10 nM Sfp and were incubated at 37°C for 10–30 min. The reaction was stopped, and the protein was precipitated by the addition of trichloroacetic acid (final concentration 10%). The amount of holo-ACP and AcpK formed was determined by an HPLC method. This method was adapted from a method described previously (15). Reaction mixtures (800 μl each) were, after the addition of trichloroacetic acid, centrifuged for 30 min at 13,000 rpm and 4°C . The supernatant was discarded, and the protein pellet resuspended in 120 μl of 50 mM Tris/HCl, pH 8.8. A 100- μl sample of this solution was injected onto an analytical Nucleosil 250 C₁₈-column (reversed phase; Macherey & Nagel) that had been equilibrated with 0.1% trifluoroacetic acid. Absorbance at 220 nm was monitored. The column was eluted with a 1.2-ml linear gradient to 60% solvent B (methanol in 0.1% trifluoroacetic acid) followed by a 7.2-ml linear gradient to 100% solvent B at 0.3 ml/min. Under these conditions, holo-ACP migrated faster than apo-ACP (24.3 and 25.2 min, respectively). The amount of holo-ACP formed was determined by comparing the peak area of the holo-ACP formed with those of both apo- and holo-ACP and subtracting the amount of holo-ACP that was already present after the heterologous expression of the protein in *E. coli* (see "Results"). Apo-AcpK and holo-AcpK were separated in a similar manner using the same column and a 15-ml linear gradient to 80% solvent C (isopropyl alcohol in 0.1% trifluoroacetic acid) followed by a 0.6-ml linear gradient to 95% solvent C at 0.3 ml/min. Holo-AcpK and apo-AcpK eluted at retention times of 53.9 and 54.8 min.

To determine K_m and k_{cat} values of AcpS and Sfp for apo-ACP and apo-AcpK, reaction mixtures (in triplicate) contained 1 mM CoA, 2–200 μM apo-ACP, or 2–60 μM apo-AcpK and either 5.6 nM AcpS or 10 nM Sfp. For the determination of the K_m value of AcpS for CoA, the reaction conditions were the same as described above except that the concentration of apo-ACP was kept at 200 μM , and concentrations of CoA were 2–500 μM . The formation of holo carrier protein was measured by the HPLC method in all cases. Carrier proteins were dialyzed before the assay against the respective assay buffer (50 mM Tris/HCl, pH 8.8, in the case of AcpS and 75 mM MES, pH 6.0, in the case of Sfp).

RESULTS

Construction of an *E. coli* acps Disruption Mutant as a Genetic Tool—Since *acps* is an essential gene in *E. coli* (20), a disruption mutant can only be generated when a complementing gene is present *in trans*. We therefore decided to first integrate a second copy of *acps* in another locus of the *E. coli* chromosome and then disrupt the gene at its natural locus (at 58 min; see Fig. 2A) with a *kan*⁺ cassette. The *acps::kan*⁺ genotype was then transduced using P1 phage into other *E. coli* strains carrying *in trans* a PPTase gene to be tested for *acps* complementation activity. For manipulations of the chromosome of *E. coli*, we used a *polA* mutant strain, HSK 42, that is unable to replicate ColE1-based plasmids and thus allows selection of integration into the chromosome. Double crossover integrations were identified by marker loss and PCR analysis with flanking primers. *acps* is the second gene in a bicistronic operon with *pdxJ* (the former name of *acps* was *dj*, for downstream of *pdxJ*) and is followed by a termination loop (15, 20). A disruption of *acps* was therefore not expected to exert polar effects on neighboring genes. The *nrdD* locus at 96 min (see Fig. 2A) was chosen for the integration of a second copy of the *acps* gene into the *E. coli* chromosome. *nrdD* encodes the anaerobic deoxyribonucleotide reductase, which is not essential under the conditions used here (21). Transformation of HSK42 with the disruption plasmid pTrc99a-5'-*acps::kan*-3' and selection on LB plates with kanamycin yielded Km^r transformants, which were exclusively found after restreaking to be also Ap^r and thus had integrated the plasmid only by a single crossover event, leaving the original *acps* gene intact. This finding confirmed the essential nature of the *acps* gene and the necessity to first introduce a second copy of the gene. To this end, HSK42 was first transformed with the integration plasmid pUC18-*nrdD::acps-spc*, and transformants were selected on LB plates with spectinomycin. About 10% of these were candidates for double crossover integration, since they were Ap^r. This genotype was confirmed by PCR using oligonucleotides E1 and E2. Only the fragment *nrdD::acps-spc*⁺ and not the wild-type fragment *nrdD* was obtained. The latter was obtained in the control using chromosomal DNA of HSK42. One transformant, HM0139 (see Table II for a list of strains), was chosen for further work and transformed with plasmid pTrc99a-5'-*acps::kan*-3' to disrupt the *acps* gene. Transformants selected on LB plates containing kanamycin were subsequently tested for Ap^r. Now candidates for double crossover integration were obtained and could be confirmed by PCR using oligonucleotides E3 and E4. One of the thus identified strains, HM0145 (relevant genotype *acps::kan*⁺, *nrdD::acps-spc*⁺; see Table II), was chosen for further work. A P1 phage lysate was prepared from HM0145, which served for transduction of the *acps::kan*⁺ genotype into *E. coli* strains carrying PPTase genes *in trans*.

Identification of the *ycdB* Gene Encoding AcpS of *B. subtilis* and Its Genetic Characterization—At the beginning of this work, the gene encoding the ACP synthase (AcpS) of *B. subtilis* was not identified or characterized. By homology searches, we identified *ycdB*, which was revealed by the *B. subtilis* genome sequencing project (22) as the putative gene coding for AcpS (33% identity to *E. coli* AcpS). *ycdB* is located at 44° of the *Bacillus* genome (see Fig. 2B), 366 bp in length, and encodes a protein of 121 aa (13.7 kDa).

We assumed that AcpS of *B. subtilis* should complement the corresponding activity in *E. coli* and therefore attempted to disrupt *acps* of *E. coli* expressing *ycdB* *in trans*. For this purpose, *E. coli* K-12 strain MG1655 was transformed with pTZ18R-*ycdB* to give strain HM0172. To test for complementation activity, the *acps::kan*⁺ genotype was then transduced with the P1 lysate of HM0145, and transductants were selected

TABLE II
Strains used in this study

Strain name	Relevant genotype or properties	Origin and reference
<i>E. coli</i>		
MG1655	<i>E. coli</i> K-12 wild type strain	<i>E. coli</i> genetic stock center
HT253	<i>pdxJ8::ΔTn10</i>	Ref. 20
HSK42	MC4100, <i>polA</i>	Ref. 39
M15	pREP4 (<i>kan</i> ⁺), expression strain	Qiagen
HM0139	HSK42, <i>nrdD::acps-spc</i>	This work
HM0145	HM139, <i>acps::kan</i>	This work
HM0169	MG1655, pTZ18R	This work
HM0170	MG1655, pUC8-sfp	This work
HM0171	MG1655, pTZ19-gsp	This work
HM0172	MG1655, pTZ18-ydcB	This work
HM0403	M15, pQE60-acpK	This work
RF1	M15, pQE60-acpA	This work
RF3	M15, pTZ18-ydcB	This work
RF4	M15, pQE60-dltC	This work
<i>B. subtilis</i>		
MR168	Wild type strain, <i>sfp0</i>	Ref. 22
JH642	<i>trpC2, pheA1, sfp0</i>	Ref. 40
Mo1099	JH642, <i>amyE::mls</i>	Ref. 40
ATCC21332	Surfactin producer, Sp ⁺	Ref. 41
OKB105	JH642 transformed with chromosomal DNA of ATCC21332, <i>sfp</i> ⁺ , <i>trp</i> ⁺ , <i>pheA1</i> , surfactin producer	Ref. 11
HM0451	Mo1099, <i>amyE::ydcB-cat</i>	This work
HM0489	OKB105, <i>ΔydcB::spc</i>	This work
HM0492	HM0451, <i>ΔydcB::spc</i>	This work
HM0491	OKB105, <i>ΔydcB::spc</i> single crossover, <i>ydcB</i> ⁺	This work

on LB plates containing ampicillin and kanamycin. Indeed, Km^r colonies could be obtained overnight using HM0172 as recipient strain, whereas no Km^r-colonies appeared in the control (strain HM0169, MG1655 with plasmid pTZ18R) under these conditions. The Km^r colonies were Sp⁺, indicating that the *nrdD::acps-spc*⁺ genotype was not co-transduced. Both genotypes were confirmed by PCR (not shown).

Sfp and Other Members of the Sfp Family Can Also Complement AcpS of *E. coli* in Vivo—We also tested the ability of PPTases of the Sfp type to modify noncognate ACP substrates under heterologous *in vivo* conditions. To this end, plasmids pUC8-sfp and pTZ19-gsp were transformed into *E. coli* K-12 to give the strains HM0170 and HM0171, respectively. As described above, the P1 lysate of HM0145 was then used to transduce the *acps::kan*⁺ genotype. In both cases, using HM0170 and HM0171, Km^r transductants (which were Sp⁺) were obtained overnight, whereas no Km^r colonies could be observed with the control strain HM0169. Further analysis of the transductants by PCR confirmed their *acps::kan*⁺ genotype. Thus, the PPTases Sfp and Gsp of secondary metabolism from *B. subtilis* and *Bacillus brevis* can also complement AcpS of *E. coli*'s primary metabolism.

Interestingly, Km^r and Sp⁺ colonies also appeared in the experiments using control strain HM0169 after a prolonged time period (after 2–3 days at 37 °C). These colonies were confirmed by PCR to be true transductants, since they were *acps::kan*⁺. Obviously, the same suppressor mutations that complement the lethal *acps* disruption were selected as previously described by Lam *et al.* for a conditional mutant of the *acps* gene (formerly *dpp*) under nonpermissive conditions (20, 23). In agreement with the results by these authors, most of the transductants displayed a very mucous morphology on LB plates. Suppressor mutations can occur in the *lon* gene and at another location in the chromosome (20, 23), presumably in the gene *yhhU* encoding a PPTase of unknown function (3).

Overproduction and Purification of *B. subtilis* AcpS, ACP, AcpK, and DCP—Pure *B. subtilis* AcpS was obtained by heterologous overexpression of the *ydcB* gene in *E. coli* and subsequent four-column purification as described under "Experimental Procedures." ACP, DCP, and AcpK (see below) of *B. subtilis*

were produced as C-terminal His₆ tag fusion proteins and purified by affinity chromatography. SDS-polyacrylamide gel electrophoresis analysis (not shown) showed that two bands were obtained in the case of ACP and DCP, pointing to partial apo to holo conversion by *E. coli* AcpS during heterologous expression. In contrast to a report on the isolation and cloning of the *acpA* gene in *E. coli* (24), neither the production of ACP nor of the other proteins seemed to significantly inhibit growth of *E. coli* cells.

Biochemical Characterization of *B. subtilis* AcpS—To determine the catalytic activity of *B. subtilis* AcpS, an HPLC assay was applied. The recombinant substrate ACP of *B. subtilis* was determined by the HPLC method to be present in a ratio of 84% apo to 16% holo form after heterologous production in *E. coli*. Kinetic constants were determined through a Michaelis-Menten fit of the data sets (see Fig. 3 and Table III). Interestingly, first saturation occurred between 2 and 8 μM apo-ACP (Fig. 3A), but velocity values began to increase when the apo-ACP concentration was raised to 20 μM apo-ACP. Therefore, two different *K_m* and *k_{cat}* values could be determined. The first *K_m* for apo-ACP concentrations between 2 and 8 μM was 0.2 ± 0.3 μM, with the *k_{cat}* being 22 ± 2 min^{−1} (Fig. 3A), and the second *K_m* was determined as 68 ± 11 μM with a *k_{cat}* of 125 ± 9 min^{−1} for apo-ACP concentrations between 20 and 200 μM (Fig. 3B). The *K_m* of AcpS for CoA was determined in essentially the same fashion, except that the apo-ACP concentration was kept constant at 200 μM, while the CoA concentration was varied between 5 and 500 μM. The Michaelis-Menten fit of the experimental data set yielded a *K_m* of 5.4 ± 1.5 μM and a *k_{cat}* of 109 ± 5 min^{−1} (Fig. 3C).

Protein Partners of AcpS and Sfp: ACP, PCP, and DCP—Since Sfp was reported to be of broad specificity, we assumed that it would also modify the ACP of *B. subtilis*. However, this experiment was intriguing, because a higher degree of specialization would be conceivable for the homologous substrates of the same organism. All cases of broad specificity of Sfp were demonstrated for heterologous acyl carrier proteins. Nevertheless, as shown in Fig. 4, Sfp also efficiently recognizes and modifies ACP of *B. subtilis*. The dispensable PPTase Sfp of the secondary metabolism can thus convert the ACP of primary

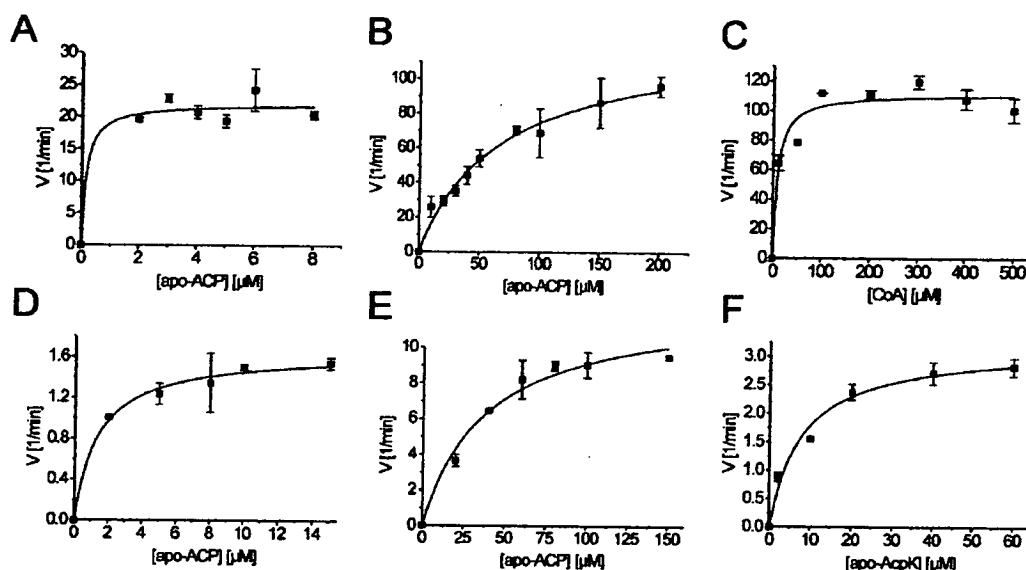


FIG. 3. Determination of kinetic constants of *B. subtilis* AcpS for its substrates apo-ACP and CoA and of Sfp for apo-ACP and apo-AcpK. Reaction mixtures were incubated for 10 min in the case of AcpS (5.6 nM) and 30 min in the case of Sfp (10 nM). For the fit of the kinetic data, a hyperbolic Michaelis-Menten function was used. The kinetic constants toward the carrier proteins are summarized in Table III. A, plot of velocity of AcpS against apo-ACP concentration between 2 and 8 μM ; the CoA concentration was held constant at 1 mM. B, when the apo-ACP concentration was further increased beyond 20 μM , a second K_m value of AcpS for apo-ACP could be determined. C, the K_m value of AcpS for CoA was determined by varying the CoA concentration between 5 and 500 μM with the apo-ACP concentration being constant at 200 μM . D and E, kinetic data for Sfp with apo-ACP concentrations between 2 and 15 μM (D) and between 20 and 150 μM (E) apo-ACP. F, K_m values for Sfp with apo-AcpK were measured between 2 and 60 μM apo-AcpK.

TABLE III
Kinetic constants of *B. subtilis* AcpS and Sfp toward ACP and AcpK

Substrate	K_m		k_{cat}		k_{cat}/K_m	
	AcpS	Sfp	AcpS	Sfp	AcpS	Sfp
	μM		min^{-1}		$\text{min}^{-1} \mu\text{M}^{-1}$	
Apo-ACP (2–8 μM)	0.2 ± 0.3	1.4 ± 0.3	22 ± 2	1.7 ± 0.1	129	1.2
Apo-ACP (20–200 μM)	68 ± 11	38 ± 8	125 ± 9	12.5 ± 1.0	1.8	0.3
Apo-AcpK	ND ^a	7.9 ± 2.1	ND	3.2 ± 0.2	ND	0.4

^a ND, not determined.

metabolism into its active holo form *in vitro*. Determination of kinetic constants revealed saturation at low and high apo-ACP concentration as observed for AcpS. In the ACP range of 2–8 μM , a K_m of $1.4 \pm 0.3 \mu\text{M}$ and a k_{cat} of $1.7 \pm 0.1 \text{ min}^{-1}$ were determined. For ACP concentrations from 20 to 150 μM , the K_m was found to be $38 \pm 8 \mu\text{M}$ with a k_{cat} of $12.5 \pm 1.0 \text{ min}^{-1}$ (see Fig. 3, D and E, and Table III). The values for low ACP concentrations are in good agreement with those determined for the interaction of Sfp with *E. coli* ACP (K_m of 6 μM and k_{cat} of 5.8 min^{-1}) (5).

We next tested the D-alanyl carrier protein DCP and a PCP for their ability to serve as substrates for AcpS and/or Sfp. Therefore, the *dltC* gene encoding DCP was cloned and expressed as a His₆ tag fusion. As a representative of PCPs, we chose the excised TycC3-PCP domain of the multimodular tyrocidine NRPS (19). As shown in Fig. 4, Sfp recognized both acyl carrier proteins as substrate, whereas AcpS could only modify DCP.

Identification of a Second ACP in *B. subtilis*, AcpK, Which Is Only Modified by Sfp—The *B. subtilis* genome project has confirmed the presence of the previously described gene *acpA* coding for ACP of fatty acid synthesis (22). We have reexamined the regions of the genome that harbor the clusters for secondary metabolite production in a search for possible misannotations of the genome project data. The *pksX* cluster contains several genes that encode enzymes homologous to fatty acid or polyketide synthases of type II (*pksB-D*), to polyketide synthases of type I (*pksKLMRP*), and to nonribosomal peptide

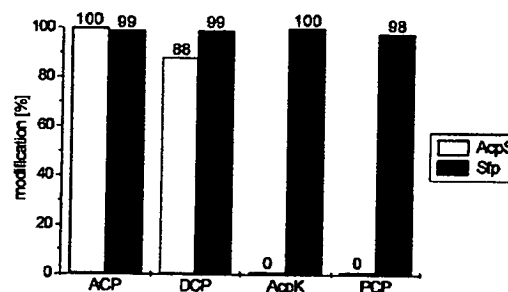


FIG. 4. Protein partners of AcpS and Sfp. The different acyl carrier proteins (6 μM) of primary and secondary metabolism of *B. subtilis* were incubated with [³H]CoA and AcpS (0.22 μM) or Sfp (0.8 μM). Shown is the modification in percent of the respective acyl carrier protein by AcpS (white column) and Sfp (black column) in a qualitative analysis after 30 min of reaction time. Sfp recognizes all acyl carrier proteins tested, whereas AcpS only modifies ACP and DCP of primary metabolism and not AcpK and PCP of secondary metabolism.

synthetases (*pksJKNR*) (25) (and GenBankTM accession number Z99113). A relatively large gap between *pksE* and *pksF* led us to reexamine this region in detail (see Fig. 5A). Surprisingly, we detected an open reading frame, thereafter designated *acpK*, that has a 20-bp overlap with the 5'-end of *pksF*. The putative gene product AcpK (82 aa, 9.251 kDa, pI 4.2) showed significant similarities to ACPs, in particular around the conserved serine residue, which serves as the Ppant attachment site (see Fig. 5B). The highest similarity was found to two

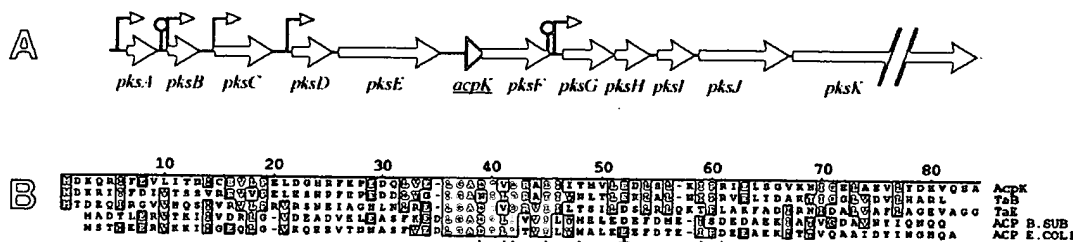


FIG. 5. Identification of a second acyl carrier protein in *B. subtilis*, designated AcpK. A, localization of the *acpK* gene in the *pksX* cluster of *B. subtilis*. Putative promoters and termination loops are indicated as suggested by Ref. 22. B, alignment of AcpK with the acyl carrier proteins TaB and TaE involved in synthesis of the antibiotic TA and with the acyl carrier proteins involved in fatty acid synthesis of *E. coli* and *B. subtilis*. The asterisks mark the residues that provide contacts between AcpS and ACP of *B. subtilis* (9).

ACPs, TaB and TaE, that are obviously involved in synthesis of the antibiotic TA in *Myxococcus xanthus* (53 and 33% identity) (26). The similarity to *B. subtilis* and *E. coli* ACPs was only 18 and 22%, respectively. Fig. 5B shows an alignment of AcpK with these acyl carrier proteins. We were interested to know whether AcpK is a substrate for AcpS or Sfp or even for both. As shown in Fig. 4, only Sfp, but not AcpS, was capable of converting AcpK into its holo form. Determination of kinetic constants revealed a normal Michaelis-Menten behavior with a K_m of $7.9 \pm 2.1 \mu\text{M}$ and a k_{cat} of $3.2 \pm 0.2 \text{ min}^{-1}$ (see Fig. 3F and Table III).

Deletion Mutant of *B. subtilis* *acps*—The gene *ycdC* (363 bp) is predicted to be organized in an operon with the downstream gene *ycdC* (1,119 bp) encoding a putative protein (42.2 kDa) of unknown function. It is not known whether *ycdC* is essential or not. We decided to attempt deletion of *ycdC* regardless of possible polar effects on *ycdC*. For the deletion, we followed a similar strategy as for *E. coli* *acps*. First, a second copy of *ycdC* was introduced into the *amyE* locus of the *B. subtilis* chromosome (see Fig. 2B) by transforming *B. subtilis* Mo1099 with pDR67-*ycdC* and selecting for Cm^r transformants. These were subsequently checked for MLS^r by restreaking. One of the Cm^r and MLS^r colonies, HM0451, was chosen for further work. HM0451 was then transformed with a PCR product containing 5' and 3' regions of the original *ycdC* gene flanking a *spc*⁺ cassette that substituted the deleted *ycdC* (obtained by PCR amplification using pTZ18-5'-*AydcB*::*spc*-3' as template; see Fig. 6B). By using the PCR product, only a double crossover recombination, and thus a deletion of *ycdC*, can lead to Sp^r transformants. *B. subtilis* MR168, JH642, and Mo1099, which are all *sfp*⁰, were transformed with this PCR product for comparison. As expected, Sp^r transformants were only obtained using HM0451, and one of these was named HM0492 (see Fig. 6 for confirmation of genotype by PCR), but not for MR168, JH642, and Mo1099. In control experiments using the circular plasmid pTZ18-5'-*AydcB*::*spc*-3' for the transformation, high numbers of Sp^r transformants were observed for all strains. However, PCR analysis of several of these transformants confirmed that they all resulted only from a single crossover event, leaving the *ycdC* gene intact (see Fig. 6). Importantly, the successful deletion of *ycdC* in strain HM0492 showed that an eventual polar effect on the downstream gene *ycdC* is not lethal.

Since Sfp was shown in this study to *in vitro* modify ACP of *B. subtilis*, we speculated that *ycdC* might not be essential in *sfp*⁺ strains. *B. subtilis* OKB105 (11) was chosen as a test strain expressing the intact *sfp* gene at physiological levels (another prominent *sfp*⁺ strain, the surfactin producer ATCC21332, was found to be already Sp^r). OKB105 was transformed with the PCR product as described above. Indeed, Sp^r transformants could be obtained. Several of these strains were chosen for PCR analysis, which confirmed their *ycdC*::*spc*⁺ genotype (see Fig. 6). One of these strains was designated

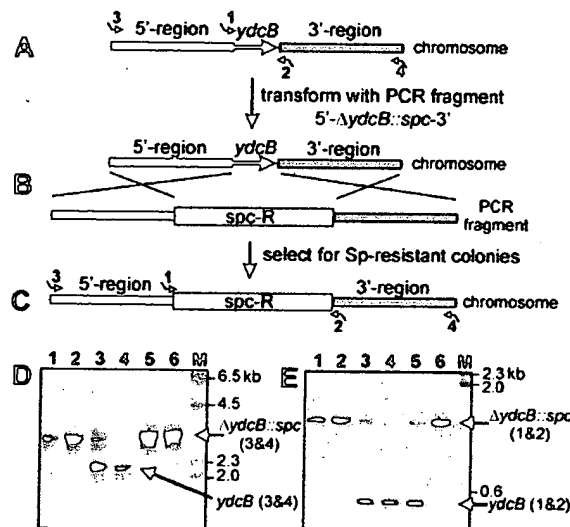


FIG. 6. Deletion of the *ycdC* gene encoding AcpS in *B. subtilis*. A, chromosomal organization around the *ycdC* gene in wild-type *B. subtilis* OKB105. A spectinomycin resistance cassette (*spc*⁺) was cloned between the 5'- and 3'-flanking regions of the *ycdC* gene (plasmid pTZ18-5'-*AydcB*::*spc*-3'). This plasmid served as a template to amplify the PCR fragment shown using oligonucleotides 3 and 4, which was then used for transformation. Double crossover recombination resulted in deletion of the *ycdC* gene from the chromosome (B). Selection of Sp^r transformants yielded strain HM0489 (*AydcB*::*spc*⁺) (C). D and E show the PCR analysis from chromosomal DNA of HM0489 and various controls: HM0489 (lane 1); HM0490 (a second *ycdC* deletion strain isolated) (lane 2); HM0491, resulting from transformation of OKB105 with circular plasmid pTZ18-5'-*AydcB*::*spc*-3', which integrated with a single crossover recombination (lane 3); OKB105 (lane 4); HM0492, carrying a second copy of *ycdC* in the *amyE* site (compare Fig. 2) (lane 5); and plasmid pTZ18-5'-*AydcB*::*spc*-3' (lane 6). D shows the PCR analysis using oligonucleotides 3 and 4. The *ycdC* wild-type locus yields a 2.1-kb PCR amplificate, and the *AydcB*::*spc*⁺ locus gives a 2.9-kb fragment. E shows the PCR analysis using oligonucleotides 1 and 2. The intact *ycdC* gene yields a 0.4-kb PCR fragment, and the *AydcB*::*spc*⁺ locus results in amplification of the 1.2-kb spectinomycin cassette.

HM0489. HM0489 still produced surfactin in amounts similar to the parent strain OKB105, as judged by analysis of hemolytic activity on blood agar plates (data not shown). Furthermore, the growth curve patterns of OKB105 and HM0489 were indistinguishable in both rich and minimal (glucose) media (data not shown). These results prove that *ycdC* is dispensable in *sfp*⁺ strains. The PPTase of secondary metabolism in *B. subtilis* can substitute for AcpS of primary metabolism under physiological conditions.

DISCUSSION

We have investigated the phosphopantetheinylation reaction in Ppant-dependent pathways of primary and secondary metabolism of *B. subtilis*. This organism employs many acyl car-

erations, it seems plausible to hypothesize that the Sfp family evolved from the AcpS family by a gene fusion event with subsequent diversification of the two halves. This evolution probably took place when the acyl carrier proteins were fused with the other enzymatic units to become integrated domains of FASs, PKSs, or NRPSs. The new architecture of the resulting large protein templates presumably was inaccessible to AcpS for steric or electrostatic reasons, as was suggested from structural data (9). Another explanation could be the need for a well defined regulation of the CoA pool (35), possibly to selectively switch on and off primary and secondary metabolism. This argument could still be valid for *E. coli*, where Ppant transfer of primary and secondary metabolism are separated, but seems irrelevant for *B. subtilis* in the light of our results. If regulatory aspects are not crucial, then one could expect that PPTases of the AcpS type should gradually get lost from strains also harboring an Sfp type enzyme, as we have experimentally simulated with the *acps* deletion mutant of *B. subtilis*. In fact, such strains can be encountered in the pool of microorganisms whose complete genome sequence has been determined. The entire genome of the Gram-negative *Pseudomonas aeruginosa*, for example, obviously contains only one PPTase encoding gene, whose gene product belongs to the Sfp type (242 aa, 12% identical to Sfp, accession number AAG04554). The *Pseudomonas* genome is rich in ACPs and NRPSs. The cyanobacterium *Synochocystis* PCC6803 also seems to lack an enzyme of the AcpS type but contains one of the Sfp type (246 aa, 21% identical to Sfp, accession number 1001183). Strikingly, however, this organism is obviously devoid of any secondary metabolism genes encoding PKSs or NRPSs, since we could only detect the homologue to ACP. The genome of *Bacillus halodurans* reveals, similar to *B. subtilis*, one PPTase gene of each group, encoding an AcpS and a Sfp homologue (119 and 214 aa, accession numbers BAB04327 and BAB05571, 48 and 21% identical to AcpS and Sfp, respectively), but again, the fatty acid ACP seems to be the only acyl carrier protein present.

We note that the wide distribution and the ability of PPTases of the Sfp type to complement the essential function of AcpS *in vivo* may pose significant problems in approaches to direct new inhibitors against AcpS, which was proposed as an attractive antimicrobial target (9, 10). Therefore, a potential inhibitor would need to be of broad enough specificity toward both types of PPTases.

The role of AcpK in *B. subtilis* is unknown, but a function in polyketide assembly can be deduced from its location within the large *pksX* cluster (see Fig. 5). There are striking similarities between the *pksX* cluster, which is completely uncharacterized except for its sequence, and the reported parts of the cluster responsible for the biosynthesis of the antibiotic TA in *M. xanthus* (26, 36). Not only does AcpK display the highest similarities to the two ACPs, TaB and TaE, but also PksG is highly similar to TaC and TaF, all of which are predicted from sequence analysis to encode 3-hydroxy-3-methyl-glutaryl-CoA synthases. Furthermore, the enzyme PksK, a mixed NRPS/PKS, has an identical domain organization (T-C-A-T-PKS) as the fragment that is known from the enzyme TA1 (-C-A-T-PKS; the N terminus has not yet been determined) (26). We found that the signature sequences of the A-domains of both NRPS enzymes are almost identical and are predicted to confer glycine specificity (37), which would fit well with the glycine residue found in the antibiotic TA. If this analogy extends further, one could speculate that the starter in the biosynthesis of the *pksX* product is the same as in antibiotic TA (26). At present, however, it cannot be ruled out that AcpK is the donor of the fatty acid for the biosynthesis of surfactin and fengycin.

Another candidate for this role would be the fatty acid ACP itself, since the fatty acid moiety of these latter antibiotics is an intermediate in fatty acid biosynthesis.

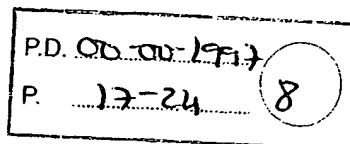
In conclusion, the many acyl carrier proteins in *B. subtilis* are converted into their active holo form by only two PPTases, AcpS and Sfp. Obviously, in this Gram-positive bacterium, a strict separation of the different biosynthetic pathways on the level of Ppant transfer, as reported for *E. coli*, is not necessary. AcpS is not essential for cell survival, because Sfp of secondary metabolism can complement its function in primary metabolism.

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Ability of *Streptomyces* spp. acyl carrier proteins and coenzyme A analogs to serve as substrates *in vitro* for *E. coli* holo-ACP synthase

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Introduction: The polyketide natural products are assembled by a series of decarboxylation/condensation reactions of simple carboxylic acids catalyzed by polyketide synthase (PKS) complexes. The growing chain is assembled on acyl carrier protein (ACP), an essential component of the PKS. ACP requires posttranslational modification on a conserved serine residue by covalent attachment of a 4'-phosphopantetheine (P-pant) cofactor to yield active holo-ACP. When ACPs of *Streptomyces* type II aromatic PKS are overproduced in *E. coli*, however, typically little or no active holo-ACP is produced, and the ACP remains in the inactive apo-form.

Results: We demonstrate that *E. coli* holo-ACP synthase (ACPS), a fatty acid biosynthesis enzyme, can catalyze P-pant transfer *in vitro* to the *Streptomyces* PKS ACPs required for the biosynthesis of the polyketide antibiotics granaticin, frenolicin, oxytetracycline and tetracenomycin. The catalytic efficiency of this P-pant transfer reaction correlates with the overall negative charge of the ACP substrate. Several coenzyme A analogs, modified in the P-pant portion of the molecule, are likewise able to serve as substrates *in vitro* for ACPS.

Conclusions: *E. coli* ACPS can serve as a useful reagent for the preparation of holo-forms of *Streptomyces* ACPs as well as holo-ACPs with altered phosphopantetheine moieties. Such modified ACPs should prove useful for studying the role of particular ACPs and the phosphopantetheine cofactor in the subsequent reactions of polyketide and fatty acid biosynthesis.

Introduction

Polyketide antibiotics are synthesized either by enzymes of very high molecular weight with multiple catalytic domains in a single polypeptide chain, type I synthases (exemplified by 6-deoxyerythronolide B synthase), or by a multienzyme complex in which the required catalytic activities reside on discrete subunits, the type II synthases (exemplified in *Streptomyces* species aromatic polyketide biogenesis) [1,2]. In both cases, the growing carbon skeleton is elongated as an acyl-S-enzyme species where the sulfur is part of the 4'-phosphopantetheine prosthetic group attached to an acyl carrier protein (ACP) module. In type I polyketide synthases (PKSs), the ACP module is an integral, constituent domain of a larger multidomain polypeptide. In type II synthases, there is a separate ACP of 8–10 kDa that associates with the other enzymatic subunits of the synthase complex (Fig. 1). Thus, in *Streptomyces* spp. producing multiple aromatic polyketides there will be a separate, dedicated ACP for each gene cluster expressed during antibiotic production [2]. These polyketide-specific ACPs are additional to the ACP involved in fatty acid biosynthesis in these bacteria [3,4]. It has been

unknown to what extent utilization of a specific ACP is determined by protein-protein recognition in type II PKS complex assembly or by selective posttranslational conversion of an inactive apo-ACP to the phosphopantetheinyl-containing holo-ACP, which is competent for acylation and chain elongation.

There have been mixed reports on whether *Streptomyces* type II PKS apo-ACPs can be posttranslationally modified to produce holo-ACP during heterologous expression in *Escherichia coli* [5,6]. If such holo-ACP formation by the *E. coli* phosphopantetheinyl transferases is of very low stoichiometry, it will be a limiting factor in polyketide biosynthesis in *E. coli*. We have recently reported the cloning, overproduction, purification and characterization of the *E. coli* enzyme holo-acyl carrier protein synthase (ACPS) that catalyzes apo to holo posttranslational modification from cosubstrate coenzyme A (CoASH), using the fatty acid apo-ACP as substrate (Fig. 2) [7]. *E. coli* ACPS is thus a useful reagent with which to assess the ability to recognize and modify *Streptomyces* type II PKS ACPs. Here, we report the ability and catalytic efficiency of

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Key words: acyl carrier protein, holo-ACP synthase, phosphopantetheine, phosphopantetheinyl transferase, polyketide synthase

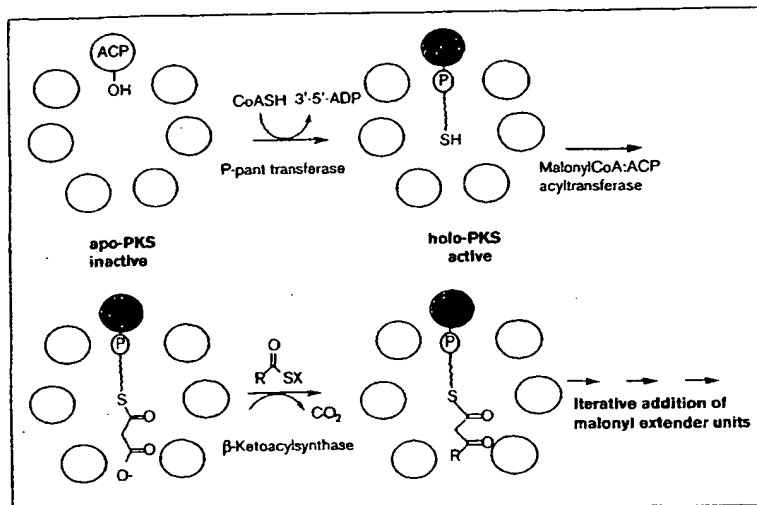
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Figure 1



Schematic of the requirement of ACP phosphopantetheinylation for polyketide synthase activity. Conversion of apo-ACP (gray) to holo-ACP (blue) provides the sulfhydryl group through which the intermediates of polyketide biosynthesis are covalently attached. Yellow circles represent the other protein subunits of the type II polyketide synthase which include at least a β -ketoacyl synthase (condensing enzyme) and a chain-length determining factor, and typically also include β -keto-reductases, acyltransferases, dehydratases and cyclases. These enzymes catalyze the iterative addition of malonyl units followed by decarboxylation and condensation reactions, reductions, and cyclizations by which the growing polyketide chain is assembled and modified.

E. coli ACPS to posttranslationally modify the purified apo-ACPs involved in the biogenesis of granaticin by *S. violaceoruber*, of frenolicin by *S. roseofulvus*, of oxytetracycline by *S. rimosus*, and of tetracenomycin by *S. glaucescens* (Fig. 3). We also observe that CoASH analogs including desulfo-CoA and the dethio (carba) analog of acetyl-CoA can be loaded onto apo-ACP by the *E. coli* ACPS.

Results

Overproduction of apo-forms of *Streptomyces* spp. ACPS

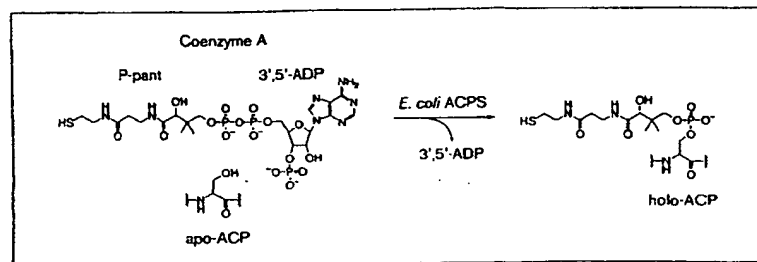
The PKS ACPS involved in the synthesis of the aromatic polyketide antibiotics granaticin (gra), frenolicin (fren), oxytetracycline (otc), and tetracenomycin (tcm; see Fig. 3) were overproduced in *E. coli*. The tcm ACP was overproduced with a carboxy-terminal hexahistidine (His₆) tag; all the other ACPS were expressed in their native form. Overexpression of these *Streptomyces* ACPS in *E. coli* yielded predominantly apo-ACP that had not been modified by the covalent attachment of 4'-phosphopantetheine as previously reported [5,6]. Mass spectral analysis of these ACPS

following the purification step on Q-Sepharose showed no significant levels of holo-protein (data not shown). The *Streptomyces* apo-ACPS were purified in several milligram quantities to near homogeneity in two steps using anion-exchange chromatography on Q-Sepharose followed by perfusion chromatography on POROS 20HQ; the His₆-tagged tcm ACP was also further purified on a nickel-chelate resin (Fig. 4). Protein sequencing of the 10 amino-terminal residues of each ACP revealed that the amino-terminal methionine had been cleaved from gra, fren, and tcm ACP but not otc ACP.

Streptomyces apo-ACPS are modified by *E. coli* holo-ACPS

Although it has previously been shown that the gra, fren, otc and tcm ACPS are not phosphopantetheinylated well *in vivo* when overexpressed in *E. coli* [5,6], the ability of *E. coli* holo-ACPS to catalyze phosphopantetheinyl transfer to these ACPS *in vitro* has not been investigated. Since ACP phosphopantetheinylation is required for the formation of an active polyketide synthase, *E. coli* ACPS may

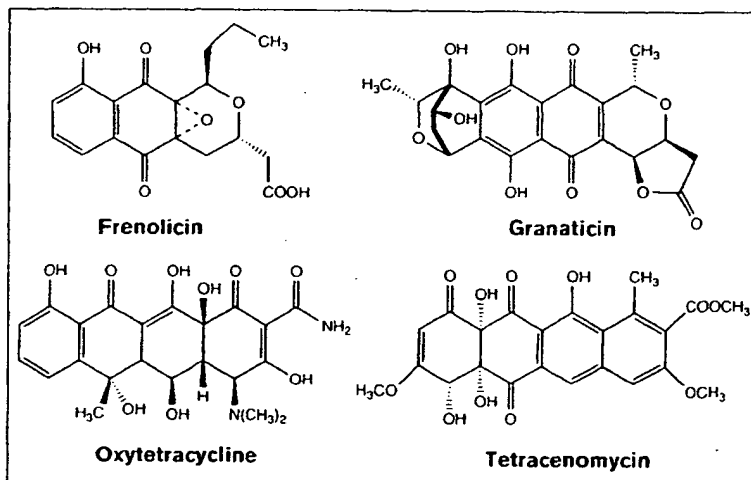
Figure 2



General reaction catalyzed by *E. coli* holo-ACPS. ACPS catalyzes transfer of phosphopantetheine (blue) from CoASH to a conserved serine residue in the apo-ACP substrate to yield 3',5' ADP (red) and active holo-ACP in a magnesium-dependent reaction.

Figure 3

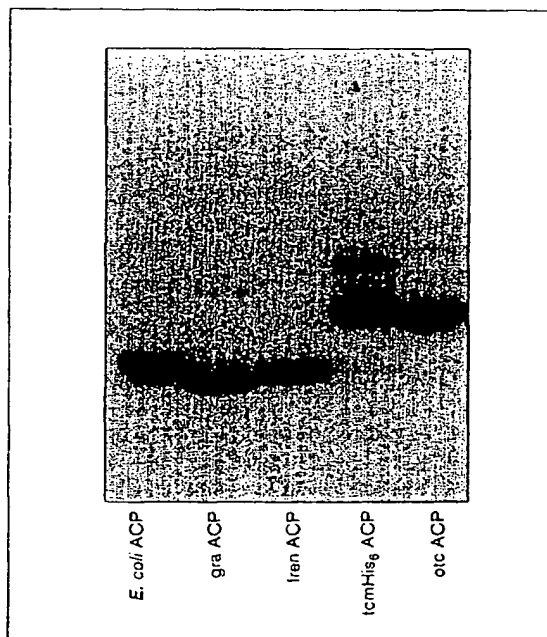
Chemical structures of selected aromatic polyketide antibiotics produced by *Streptomyces* spp. Frenolicin (fren) from *S. roseofulvus*, granaticin (gra) from *S. violaceoruber*, oxytetracycline (otc) from *S. rimosus*, and tetracenomycin (tcm) from *S. glaucescens*. Apo-ACPs from each of their respective polyketide synthases were purified (Fig. 4).



serve as a useful reagent for the preparation of holo-PKS ACPs that can be used to study downstream reactions in polyketide biosynthesis. Mass spectral data were collected for each PKS ACP following incubation for several hours with CoASH and pure ACPS. Table 1 shows that gra, fren, otc, and tcmHis₆ ACP were all modified from the apo- to the holo-form as indicated by an increase in mass of ~339 Da due to the addition of phosphopantetheine; modification appeared essentially complete, as indicated by the absence of a mass spectral peak corresponding to the apo-form. It has previously been shown that apo- and holo-forms of *E. coli* ACP [8,9] and *Streptomyces* type II ACPs [6] may be separated by high performance liquid chromatography (HPLC). Using otc ACP as a representative *Streptomyces* PKS ACP, the apo- and holo-forms were similarly resolved (Fig. 5); coinjection of otc apo-ACP and otc ACP following incubation with CoASH and ACPS shows two peaks at 32.6 min and 33.9 min, indicative of the transformation of otc ACP from the apo- to the holo-form and corroborating the mass spectral data.

To investigate the substrate specificity of *E. coli* ACPS, K_m and k_{cat} parameters were determined for each of the *Streptomyces* ACP substrates under initial velocity conditions (Table 2; Fig. 6). When gra ACP was the substrate, the reaction showed severe substrate inhibition of similar magnitude to the substrate inhibition observed with the cognate substrate of ACPS, the ACP of *E. coli* fatty acid synthase (FAS) ([17]; R.S. Flugel, V.L. Healy, R.H.L. and C.T.W., unpublished data). The severity of the observed inhibition of ACPS by gra apo-ACP made an accurate estimation of the K_m and k_{cat} values difficult; gra ACP, however, qualitatively gives the highest V_{max} and as a substrate for ACPS seems to be no more than 10-fold worse

Figure 4



Native PAGE gel (20%) demonstrating the purity of the *Streptomyces* apo-ACPs used. Approximately 10–12 μ g of each ACP was loaded. The upper band in the gra, otc and tcmHis₆ lanes most probably represents an apo-ACP dimer which has been previously reported [6], given that holo-ACP was not detected by mass spectrometry, that each of these proteins contains a single cysteine residue which is lacking in *E. coli* and fren ACP and that the intensity of this band may be diminished by incubation with DTT.

Table 1

Mass spectral data demonstrating the modification of *Streptomyces* ACPs by *E. coli* ACPS.

Substrate	Molecular mass (Da)			
	Before incubation with ACPS (apo)		After incubation with ACPS (holo)	
	Calculated	Observed	Calculated	Observed
gra ACP	8860	8863	9199	9199
fren ACP	8664	8682	9003	9009
otc ACP	9917	9927	10256	10260
tcm ACP (His ₆)	10018	10020	10357	10376

than the *E. coli* ACP. When fren ACP is used as the substrate, the substrate inhibition observed is considerably less than that for gra ACP; there is no substrate inhibition when tcmHis₆ and otc ACP are used. This attenuation of substrate inhibition is concomitant with a decrease in the catalytic efficiency of ACPS modification, with both K_m and k_{cat} kinetic parameters affected. Fren ACP is an approximately four-fold poorer substrate for ACPS than gra ACP, while otc and tcmHis₆ ACP have catalytic efficiencies decreased almost 25-fold when compared to gra ACP and about six-fold when compared to fren ACP. The poorer *Streptomyces* apo-ACP substrates are processed at a ~200-fold lower catalytic efficiency than *E. coli* FAS apo-ACP. The decrease in catalytic efficiency of a *Streptomyces* PKS ACP as a substrate for *E. coli* holo-ACP synthase appears to correlate with a decrease in the overall negative charge in the highly anionic ACP family (Table 2). It should be noted that the carboxy-terminal hexahistidine tag on the tcmHis₆ substrate may well contribute to an increase in its K_m value and/or a decrease in k_{cat} relative to the native tcm ACP substrate, which was not examined here.

Processing of CoASH analogs by holo-ACP synthase

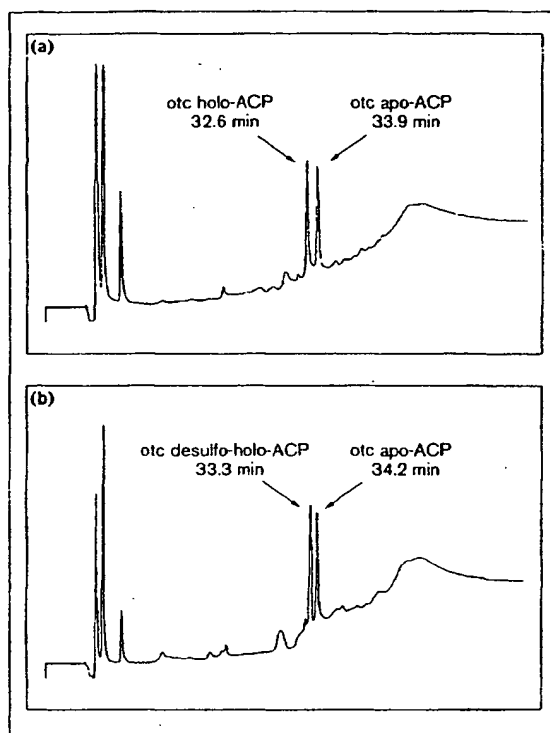
The specificity of *E. coli* holo-ACPS for its coenzyme A substrate was also examined in endpoint assays. CoASH analogs with modifications in the phosphopantetheine portion of the molecule were tested including homocysteamine-CoA, acetylthio-CoA, and desulfo-CoA (Fig. 7). ACPS could catalyze modification of *E. coli*

Table 2

Kinetic parameters for *Streptomyces* ACPs as substrates for *E. coli* ACPS.

Substrate	Charge (pH 7)	k_{cat} (min ⁻¹)	K_m (μ M)	k_{cat}/K_m (μ M ⁻¹ min ⁻¹)
<i>E. coli</i> ACP	-14.89	80-100	2 ($K_i=2$)	50
gra ACP	-14.09	≥30	≥5 ($K_i=0.6$)	6
fren ACP	-12.07	19	12 ($K_i=54$)	1.6
tcm ACP (His ₆)	-11.76	5.4	22	0.25
otc ACP	-11.10	10	39	0.26

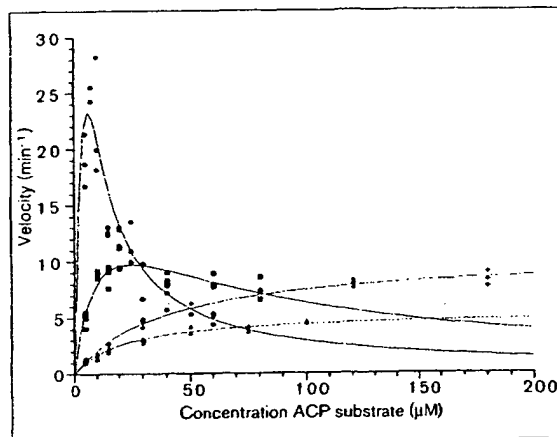
Figure 5



Conversion of otc apo-ACP to holo-ACP (220 nm, absorbance units full scale (AUFS) = 0.25). (a) Otc (50 μ M) was incubated as described in the Materials and methods section with CoA in the presence of ACPS and also in the absence of ACPS. Equal volumes (150 μ l) of the two assays were then mixed and 200 μ l of this mixture was injected onto the HPLC C18 reversed-phase column; Rt (apo-ACP) 33.9 min, (holo-ACP) 32.6 min. Single injections (data not shown) confirmed that the otc apo-ACP was quantitatively converted to the holo-form. (b) Sample was prepared as described above except otc apo-ACP was incubated with desulfo-CoA; Rt (apo-ACP) 34.2 min, (desulfo-holo-ACP) 33.3 min. Single injections confirmed that the otc apo-ACP was quantitatively converted to desulfo-holo-ACP (data not shown).

ACP with each of these phosphopantetheine analogs as determined by mass spectral analysis (Table 3) and analytical HPLC analysis (Fig. 8). Both the acetylthio-holo-ACP and the homocysteamine-holo-ACP could be well resolved from apo-ACP when coinjected onto the HPLC column (Fig. 8). The ability to use desulfo-CoA also extended to the modification of otc ACP as can be seen by its conversion to the desulfo form of the holo-protein (Fig. 5). The conversion of both the carba (dethia) analog of acetyl-CoASH and the homocysteamine form of CoASH to the holo-ACP form (Fig. 8) indicates that ACPS has relaxed specificity for the distal end of the phosphopantetheinyl moiety of CoASH, and suggests that it may be possible to load acylpantetheines directly onto apo-ACP substrates.

Figure 6



Velocity versus substrate concentration plots for the reaction of ACPs with gra (black circles), fren (blue squares), otc (pink diamonds) and tcmHis6 (purple triangles) apo-ACP. Incubation conditions were as follows: gra ACP, 2 nM ACPs for 15 min; fren ACP, 18 nM ACPs for 10 min; otc ACP, 18 nM ACPs for 15 min; tcmHis6 ACP, 18 nM ACPs for 15 min.

Discussion

Study of the type II aromatic PKSs *in vitro* requires sufficient quantities of active holo-ACP, that has been correctly posttranslationally modified by the covalent attachment of the 4'-phosphopantetheine cofactor on which the growing polyketide is then assembled. Overproduction of various type II PKS ACPs in *E. coli*, however, has yielded ACP exclusively or predominantly in the inactive, unmodified apo-form. Shen *et al.* [5] reported that overexpression of the tcm ACP in *E. coli* gives no holo-ACP as detected by electrospray mass spectrometry, ^{31}P NMR and [^3H] β -alanine labeling when cultures are induced in the exponential phase of growth; induction in the stationary phase of growth allows the recovery of a small percentage of tcm holo-ACP. Crosby *et al.* [6] examined the apo to holo ratio for gra, fren, otc and actinorhodin (act) ACP overproduced for 2–3 h in *E. coli*; about 30% and 2% of the gra and act ACPs respectively could be found in the holo-form, while

Table 3

Mass spectral data demonstrating the modification of ACPs with CoA analogs catalyzed by *E. coli* ACPs.

Substrate	CoA analog	Molecular mass (Da)	
		Calculated	Observed
<i>E. coli</i> ACP	none (apo)	8508	8513
	CoA	8847	8850
	homocysteamine-CoA	8861	8867
	acetylthio-CoA	8871	8906
	desulfo-CoA	8815	8828
otc ACP	desulfo-CoA	10224	10236

fren and otc ACP remained unmodified. For act ACP, increasing the post-induction period to 12 h allowed 80–90% of the act ACP to be obtained in the holo-form, but these fermentation conditions also led to a significant drop in the total amount of recoverable protein [6]. Thus, standard overproduction approaches have not previously led to useful quantities of posttranslationally-modified, active holo-ACP species. Given the low recovery of PKS holo-ACPs and the implied low-level recognition of these substrates by *E. coli* ACPs, we wished to determine if *in vitro* incubation of these PKS ACPs with pure *E. coli* ACPs could be used for the preparation of functional PKS holo-ACP. Indeed, as shown by the data presented here, ACPs does catalyze transfer of phosphopantetheine to gra, fren, otc and tcm ACP to give near quantitative conversion of these proteins from their apo- to holo-forms.

The PKS ACPs examined here are also of interest for delineation of the substrate specificity of the *E. coli* holo-ACP synthase. ACPs will catalyze *in vitro* phosphopantetheinyl transfer to D-alanyl carrier protein (9 kDa) from *Lactobacillus casei* involved in the D-alanylation of lipoteichoic acid [10] and also to NodF from *Rhizobium leguminosarum* (9.9 kDa) involved in the biosynthesis of a lipo-chitin oligosaccharide molecule (R.H.L., A.M.G., C.J.W., T. Ritsema and H.P. Spaink, unpublished observations). But, ACPs will not transfer phosphopantetheine to PCP (13 kDa), a peptidyl carrier protein domain derived from the type I non-ribosomal

Figure 7

Chemical structures of the coenzyme A analogs used in this study.

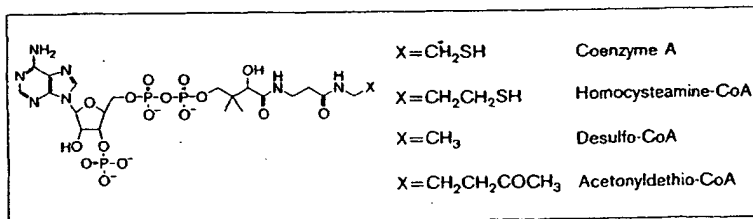
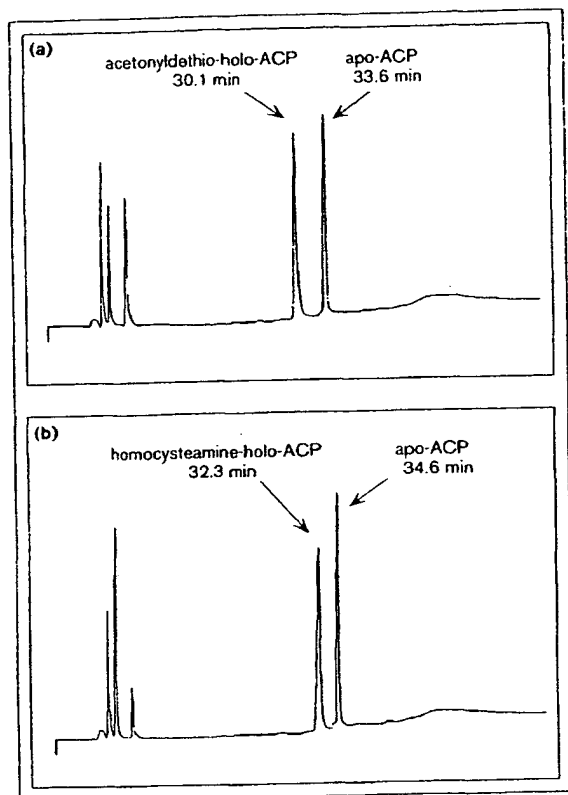


Figure 8



Modification of *E. coli* ACP with CoA analogs catalyzed by ACPS (220 nm, AUFS = 1.0). (a) Apo-ACP (500 μ M) was incubated with 125 μ M acetylthio-CoA in the presence and absence of 1.5 μ M ACPS. Equal volumes (150 μ l) of the two assays were then mixed and 200 μ l of this mixture was injected onto the HPLC C18 reversed-phase column; R_t (apo-ACP) 33.6 min, (acetylthio-holo-ACP) 30.1 min. Single injections (data not shown) confirmed that the apo-ACP was quantitatively converted to the modified holo-form. (b) Sample was prepared as described above except apo-ACP was incubated with homocysteine-CoA; R_t (apo-ACP) 34.6 min, (homocysteine-ACP) 32.3 min. Single injections confirmed that the apo-ACP was quantitatively converted to homocysteine-holo-ACP (data not shown).

peptide tyrocidine A synthetase responsible for tyrocidine production in *Bacillus brevis*. This dichotomy has led to our detection of a second class of phosphopantetheinyl transferases that recognize the apo-ACP domains in the multidomain peptide synthetases [11].

It is, therefore, of considerable interest to determine the effectiveness of ACPS as a phosphopantetheinylating agent for the ACPs involved in polyketide biosynthesis. The *gra*, *fren*, *otc*, and *tcm* ACPs have 19.5–23.4% similarity to the *E. coli* FAS ACP. The kinetic studies presented here indicate that, despite the fact that they all

have approximately the same degree of similarity to *E. coli* ACP, there is a 25-fold range in the catalytic efficiencies of these PKS ACPs as substrates for ACPS, in the order *E. coli* ACP > *gra* ACP > *fren* ACP > *tcmHis₆*, *otc* ACP, that correlates with the degree of overall negative charge of these proteins (Table 2). The more negatively-charged an ACP substrate is, the more efficient a substrate this protein appears to be for ACPS. This hypothesis is supported by kinetic data determined for the reaction of ACPS with NodF; with an overall charge at pH 7 of -10.07 , NodF gives a k_{cat}/K_m value of $0.05 \mu\text{M}^{-1} \text{min}^{-1}$ (A.M.G., C.T.W., T. Ritsema and H.P. Spaink, unpublished observations). The tyrocidine PCP substrate, with its essentially neutral charge, is not a substrate for ACPS [11,12], but as it is a 115 amino acid (aa) domain dissected from a 1087 aa type I nonribosomal peptide synthetase, structural factors may contribute to its inability to serve as a substrate. An increase in negative charge also correlates with an increased ability of an apo-ACP substrate to inhibit ACPS; both *E. coli* apo-ACP and *gra* apo-ACP are severe substrate inhibitors of ACPS, while *fren* apo-ACP is a weaker inhibitor and *tcmHis₆* and *otc* apo-ACP do not inhibit ACPS. Given the complications of kinetic analysis when substrate inhibition is a factor, *tcmHis₆* and *otc* ACP may prove more tractable substrates for further examination of the mechanism of holo-ACP synthase.

Genes encoding phosphopantetheinyl transferase(s) in *Streptomyces* spp. have not yet been identified. A crude extract from *S. coelicolor* can catalyze incorporation of phosphopantetheine into the *E. coli* ACP, but the protein(s) responsible have not been purified (A.M.G., Carreras, C., Khosla, C. and C.T.W., unpublished observations). It is not yet clear whether *Streptomyces* spp. will possess a universal phosphopantetheinyl transferase or multiple phosphopantetheinyl transferases. Multiple ACPs have been identified in *S. coelicolor* and *S. glaucescens*. In *S. glaucescens*, a constitutive ACP presumably involved in fatty acid biosynthesis is encoded by the *fabC* gene while the *tcmM* gene encodes the tetracenomycin biosynthesis ACP [3]. In *S. coelicolor* A3(2), a constitutive fatty acid ACP encoded by the *acpP* gene has also been identified which, with the ACP genes found in the *act* (actinorhodin biosynthesis) and *whiE* (spore pigment biosynthesis) PKS gene clusters, brings the number of different ACPs in this organism to three [4]. The *S. coelicolor* FAS ACP gene was unable to replace the *act* ACP gene for the production of actinorhodin *in vivo* [4], indicating the functional separation of the FAS and PKS pathways. The malonyl-coenzyme A:ACP acyltransferase in *S. glaucescens* [3] and *S. coelicolor* A3(2) [13], however, appears to function in both fatty acid and polyketide biosynthesis. It will be interesting to determine if activation of ACPs by phosphopantetheinylation represents another point of divergence and/or specificity between the FAS and PKS pathways. In *E. coli*, two and possibly three phosphopantetheinyl transferases have

been identified (ACPS, EntD, o195) each with clear specificity for their cognate substrates [11].

Significance

The feasibility of generating holo-ACPs with altered phosphopantetheine moieties has been demonstrated in this study by testing several coenzyme A analogs as substrates for ACPS. Since CoA analogs modified in the pantetheine arm are readily available, it should now be possible to routinely generate preparative quantities of holo-ACPs with phosphopantetheine groups of altered composition, length and reactivity. This opens the door to studies of the role of phosphopantetheine in the subsequent reactions of acyltransfer, condensation, reduction, dehydration and cyclization in fatty acid and polyketide biosynthesis. For example, the desulfopantetheinyl and the acetyldehiopantetheinyl holo-ACP forms may be selective inhibitors of particular steps in polyketide elongation. Similarly, in lieu of an as yet uncharacterized *Streptomyces* phosphopantetheinyl transferase, the ability of ACPS to modify the PKS ACPs studied here makes it a valuable reagent for the large-scale preparation of active PKS holo-ACP for further enzymatic studies. Also, coexpression of streptomycete apo-ACP molecules in an *E. coli* strain overproducing the *E. coli* holo-ACP synthase should yield functional holo-ACP and species competent for aromatic polyketide assembly.

Materials and methods

Overproduction and purification of *Streptomyces* apo-ACPs
Plasmids containing genes for the following *Streptomyces* PKS acyl carrier proteins were kindly provided by Chaitan Khosla, Stanford University: granaticin ACP (*S. violaceoruber*), frenolicin ACP (*S. roseofulvus*), oxytetracycline ACP (*S. rimosus*) and tetracenomycin ACP (*S. glaucescens*). These plasmids were used as the templates for PCR amplification of these genes using the following primers: gra ACP forward primer: 5'-TCCAGCTAGGATCATATGGCTCGTCTGACCCTGGACGGTCTGCG-3', gra ACP reverse primer: 5'-GATTAGTCAACAGCTTTTCAAGGCCCTCGGCCTGGGC-3'; fren ACP forward primer: 5'-ACATTCAGGATACCATATGAGCGCACTGACCGTTGACGAC-3', fren ACP reverse primer: 5'-AATGGCGAATAAGCTTTCAGCGGTGGCCGGGGTGGTGT-3'; otc ACP forward primer: 5'-GATCTCATGCACATATGACCCTGCTGACCCGTGCCGA-3', otc ACP reverse primer: 5'-AACTCGATGCAAGCTTTCACTTGTC-CGCGCGGCGCCA-3'; tcm ACP forward primer: 5'-AAGTGCATGCGACATATGCCGAGATCGGCCTGCCGCGTCTCGTC-3', tcm ACP reverse primer: 5'-GTAGCTTAGCAAGCTTTTCAAGCGACCTCCCGGCCGCTCTC-3' (all primers from Integrated DNA Technologies). The forward primers introduce an *NdeI* restriction site (italic) while the reverse primers introduce a *HindIII* restriction site (italic). In addition, the forward primers optimized the codon usage for the first eight codons of the ACP genes to facilitate expression in *E. coli*. The *NdeI/HindIII*-digested PCR product was cloned into pET22b (Novagen) and transformed into competent DH5a and BL21(DE3) *E. coli* cells. The identity of the recombinant ACP genes was confirmed by DNA sequencing (Biopolymer Laboratory, Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School). Surprisingly, DNA sequencing revealed that the tcm ACP gene had inadvertently been obtained with a carboxy-terminal His₆ tag supplied by the pET22b vector and appending the amino acid sequence LEHHHHHH to the overexpressed tcm ACP.

Cultures of BL21(DE3)pET22b-gra, BL21(DE3)pET22b-fren, BL21(DE3)pET22b-otc, and BL21(DE3)pET22b-tcmHis₆ (2 × 11, 2 × YT media) were each grown at 37°C to an optical density (O.D.) of 0.5–0.8 and then induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Growth continued for 3–4 h and cells were harvested by centrifugation. The overproduced ACPs were released and purified by a variation of the freeze/thaw method of Morris et al. [14]. The harvested cells were frozen as a cell pellet at –80°C for 24 h and then thawed in buffer A (50 mM Tris-HCl, 2 mM dithiothreitol (DTT), 2 mM EDTA, pH 7.5) to release the overexpressed ACPs without lysing the cells. This freeze/thaw supernatant was then loaded onto a Q-Sepharose column (2.5 × 10 cm) which had previously been equilibrated with buffer A. For purification of gra ACP, a 200 ml gradient of 0–0.4 M KCl (in buffer A) followed by 400 ml of a gradient from 0.4–0.8 M KCl was then applied at a flow rate of 2 ml min^{–1} with ACP eluting at ~0.4 M salt as indicated by 20% native PAGE of the column fractions. For purification of fren ACP, 150 ml of a 0–0.3 M KCl gradient (in buffer A) was followed by a 300 ml 0.3–0.6 M KCl gradient and a 150 ml 0.6–0.8 M KCl gradient with ACP again eluting at ~0.4 M salt. For otc ACP purification, the column was washed with buffer A (100 ml) and gradients of 0–0.2 M KCl (150 ml), 0.2–0.5 M KCl (300 ml) and 0.5–0.8 M KCl (50 ml) were applied (ACP elutes at ~0.28 M KCl). Finally, for tcmHis₆ ACP purification, the column was washed with 50 ml buffer A and gradients of 0–0.25 M KCl (150 ml), 0.25–0.55 M KCl (300 ml) and 0.55–0.8 M KCl (100 ml) applied (ACP elutes at ~0.35 M KCl). Fractions containing each ACP as judged by 20% native PAGE were pooled and dialyzed against 2 l buffer A. At this stage in the purification, a sample of each ACP was submitted for amino-terminal protein sequencing (10 cycles) and MALDI-TOF mass spectral analysis (Howard Hughes Medical Institute Biopolymer Facility, Harvard Medical School).

To obtain samples of each ACP purified to near homogeneity, the BioCAD™ SPRINT™ perfusion chromatography system (PerSeptive Biosystems, Inc.) was used. A portion of the dialyzed ACP samples following Q-Sepharose chromatography was applied to a POROS 20HQ anion-exchange column at a flow rate of 10 ml min^{–1}. The optimum pH for separating the ACPs from the remaining contaminants was determined to be gra ACP, pH 9.0; fren ACP, pH 6.0; otc ACP, pH 9.0; and tcmHis₆ ACP, pH 9.0. Proteins were eluted with a gradient of 0–0.5 M NaCl in 10 mM Tris, 10 mM Bis-tris propane of the indicated pH. The tcmHis₆ ACP sample was additionally purified by nickel chelate chromatography according to the manufacturer's instructions (Novagen). The buffer was exchanged for 50 mM Tris-HCl, pH 8.0 and the protein samples concentrated using Centrprep 3 (Amicon). Protein concentration was determined by measuring the absorbance at 280 nm using the following calculated extinction coefficients [15]: gra ACP 1400 M^{–1} cm^{–1}, fren ACP 2560 M^{–1} cm^{–1}, otc ACP 2680 M^{–1} cm^{–1}, and tcmHis₆ ACP 2680 M^{–1} cm^{–1}.

Preparation of the CoASH analogs

Desulfocoenzyme A was purchased from Sigma Chemical Company. Acetyldehiopantetheine A was prepared as described previously [16]. Homocysteine-coenzyme A was prepared as follows. A suspension of 0.88 g homocysteine hydrochloride [17,18] in 1 ml H₂O was added to 10 mg adenosine 5'-(trihydrogen diphosphate) 3'-(dihydrogen phosphate) 5'-[(R)-3-hydroxy-4-[[3-(propylthio)-3-oxopropyl]amino]-2,2-dimethyl-4-oxobutyl] ester [19] in 1 ml H₂O and the pH adjusted to 10 with 6 M NaOH. The reaction was stirred at room temperature while nitrogen was gently bubbled through the solution. The reaction was monitored by analytical HPLC and complete disappearance of the starting CoA synthase (R_t = 17.3 min) and appearance of product (R_t = 13.7 min) was observed in 4 h. The reaction was filtered, the pH was adjusted to 4.5, and the product partially purified by preparative HPLC (5 min 5% methanol (solvent B) followed by a linear gradient to 45% B over 40 min, product eluted at 25–32 min). The product was further purified on a DEAE-Sepharose column (20 × 2.5 cm, 0–0.2 M NaCl pH 2.8, product eluted at 0.13 M NaCl) to yield 4.5 mg homocysteine-CoA. λ_{max} = 260 nm. ¹H NMR (as potassium

salt, 400mHz, D₂O): δ 0.724 (s, 3H), 0.852 (s, 3H), 1.65–1.72 (m, 2H), 2.392 (t, 2H, $J=6.4$ Hz), 2.447 (t, 2H, $J=7.0$ Hz), 3.174 (t, 2H, $J=6.8$ Hz), 3.409 (t, 2H, $J=6.2$ Hz), 3.516 (dd, 1H, $J=4.8, 10.0$ Hz), 3.785 (dd, 1H, $J=4.8, 10.0$ Hz), 3.962 (s, 1H), 4.195 (br s, 2H), 4.551 (br s, 1H), 6.160 (d, 1H, $J=6.0$ Hz), 8.306 (s, 1H), 8.561 (s, 1H). Mass Spec HRMS (FAB): (M-H)⁺ calc'd for C₂₂H₃₇N₇O₁₆P₃S m/z 780.191, found 780.126.

Analytical and preparative-scale HPLC experiments were performed using a Perkin-Elmer 250 HPLC with a PE LC-235 diode array detector and a gradient of methanol (solvent B) in aqueous potassium phosphate (solvent A, 50mM KH₂PO₄ for analytical runs, 10mM KH₂PO₄ for preparative runs). Analytical HPLC was done on a Rainin Microsorb C18 column (4.6mm×25cm) with monitoring at 215 and 260nm. Compounds were eluted with a flow rate of 1mlmin⁻¹ with 5% solvent B for 2min, followed by a linear gradient to 60% solvent B over 12min, and then maintained at 60% solvent B. Preparative scale HPLC was done on a Rainin Microsorb C18 column (21.4cm×25cm) with monitoring at 215 and 280nm and a flow rate of 10mlmin⁻¹. Mass spectral analysis was performed at the University of California at Riverside Mass Spectrometry Facility, Riverside, CA. The concentrations of CoA analog solutions were determined using $\epsilon_{260} = 15400 \text{ M}^{-1}\text{cm}^{-1}$.

Assay for apo-ACP to holo-ACP conversion by transfer of ³H-phosphopantetheine from ³H-coenzyme A

The radioassay for determination of phosphopantetheinyl transferase activity has been previously described [7,9,11]. Briefly, in a final volume of 100 μ l, substrate (apo-gra, fren, otc or tcmHis₆ ACP) was incubated with 75mM Tris-HCl, pH 8.8, 10mM MgCl₂, 25mM DTT, 220 μ M [³H]-[pantetheinyl]-CoASH (specific activity 105 μ Ci μ mol⁻¹, 70% label in phosphopantetheine portion of the molecule) and *E. coli* holo-ACP synthase (prepared as described in [7]) at an appropriate concentration at 37°C for a specified time. Reactions were quenched with 800 μ l 10% trichloroacetic acid (TCA), bovine serum albumin (375 μ g) was added as a carrier, and protein precipitate pelleted by centrifugation. This pellet was washed 3x with 10% TCA and the pellet was dissolved in 1M Tris base. The amount of radiolabel incorporated into the solubilized protein pellet was then determined by liquid scintillation counting. For the K_m measurements of the *Streptomyces* ACPs as substrates for holo-ACP synthase, the following conditions were used: gra ACP was incubated at varying concentrations over the range 5–60 μ M with 2nM ACPS for 15min; fren ACP (range 5–80 μ M) was incubated with 18nM ACPS for 10min; otc ACP (range 5–180 μ M) was incubated with 18nM ACPS for 15min; tcmHis₆ ACP (5–100 μ M) was incubated with 18nM ACPS for 15min. The amount of tritium incorporation at each ACP concentration was determined in triplicate. Samples of each *Streptomyces* ACP were submitted for MALDI-TOF mass spectral analysis following incubation for several hours with 1.8 μ M ACPS using the same conditions described for the radioassay except that 2mM CoASH or 200 μ M desulfoCoA was used as indicated.

HPLC and mass spectrometry assay for modification of ACPs

In a 1.5-ml microcentrifuge tube, 1mM CoA (or 125 μ M CoA analog), 500 μ M apo-ACP (or 50 μ M otc apo-ACP), 20mM MgCl₂, 10mM DTT, 150mM Tris-HCl, pH 8.8, and 1.5 μ M ACPS (100 μ l final volume) were incubated at 37°C for 30min. Reactions for HPLC analysis were quenched with 900 μ l 0.1% trifluoroacetic acid (TFA). A sample (200 μ l) of this solution was injected onto a C18 reversed phase analytical column (Vydac) which had been equilibrated with 15% isopropanol in 0.1% TFA (eluant A) at 0.5mlmin⁻¹. Absorbance at 220nm was monitored. The sample was eluted from the column with 2.5ml 100% A, a 10ml linear gradient to 40% B (75% isopropanol in 0.1% TFA), followed by a 7.5-ml linear gradient to 100% B at a constant flow rate of 0.5mlmin⁻¹. Under these conditions holo-ACP elutes before apo-ACP. Samples for MALDI-TOF mass spectrometry analysis were submitted as the crude incubation mixtures (Biopolymers Facility, Howard Hughes Medical Institute, Harvard Medical School).

Acknowledgements

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A new enzyme superfamily – the phosphopantetheinyl transferases

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Background: All polyketide synthases, fatty acid synthases, and non-ribosomal peptide synthetases require posttranslational modification of their constituent acyl carrier protein domain(s) to become catalytically active. The inactive apo-proteins are converted to their active holo-forms by posttranslational transfer of the 4'-phosphopantetheinyl (P-pant) moiety of coenzyme A to the sidechain hydroxyl of a conserved serine residue in each acyl carrier protein domain. The first P-pant transferase to be cloned and characterized was the recently reported *Escherichia coli* enzyme ACPS, responsible for apo to holo conversion of fatty acid synthase. Surprisingly, initial searches of sequence databases did not reveal any proteins with significant peptide sequence similarity with ACPS.

Results: Through refinement of sequence alignments that indicated low level similarity with the ACPS peptide sequence, we identified two consensus motifs shared among several potential ACPS homologs. This has led to the identification of a large family of proteins having 12–22 % similarity with ACPS, which are putative P-pant transferases. Three of these proteins, *E. coli* EntD and o195, and *B. subtilis* Sfp, have been overproduced, purified and found to have P-pant transferase activity, confirming that the observed low level of sequence homology correctly predicted catalytic function. Three P-pant transferases are now known to be present in *E. coli* (ACPS, EntD and o195); ACPS and EntD are specific for the activation of fatty acid synthase and enterobactin synthetase, respectively. The apo-protein substrate for o195 has not yet been identified. Sfp is responsible for the activation of the surfactin synthetase.

Conclusions: The specificity of ACPS and EntD for distinct P-pant-requiring enzymes suggests that each P-pant-requiring synthase has its own partner enzyme responsible for apo to holo activation of its acyl carrier domains. This is the first direct evidence that in organisms containing multiple P-pant-requiring pathways, each pathway has its own posttranslational modifying activity.

Introduction

Multienzyme complexes exist for acyl group activation and transfer reactions in the biogenesis of fatty acids, the polyketide family of natural products (e.g. erythromycin and tetracycline), and almost all non-ribosomal peptides (e.g. vancomycin, cyclosporin, bacitracin and penicillin). All of these complexes contain one or more small proteins, ~80–100 amino acids (aa) long, either as separate subunits or as integrated domains, that function as carrier proteins for the growing acyl chain. These acyl carrier protein (ACP) domains, which may be one of the domains of a multi-functional enzyme (in the type I synthases) or a separate subunit (in the type II multienzyme complex synthases), can be recognized by the conserved sequence signature motif (L,V)(G,L)(G,A,F,Y)(D,H,K,E)S(L,Q)(D,A,G) [1]. They are converted from inactive apo-forms

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Key words: ACP, acyl carrier protein, biosynthesis, non-ribosomal peptide synthetase, phosphopantetheine

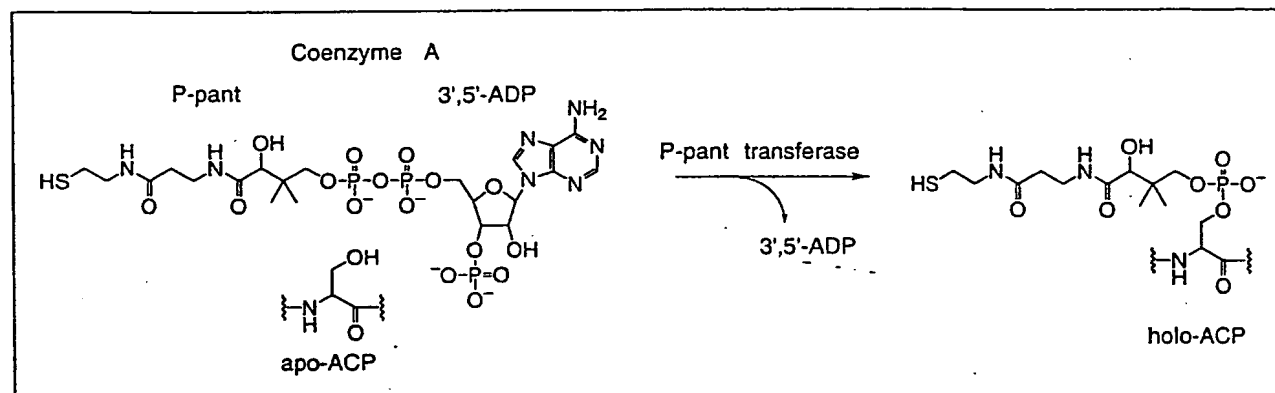
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to functional holo-forms by attack of the β -hydroxy sidechain of the conserved serine residue in the ACP signature sequence on the pyrophosphate linkage of coenzyme A (CoASH). This results in transfer of the 4'-phosphopantetheinyl (P-pant) moiety of CoASH onto the attacking serine (Fig. 1). The newly introduced -SH of the P-pant prosthetic group now acts as a nucleophile for acylation by a substrate, which may be acyl-CoA or malonyl-CoA derivatives for the fatty acid and polyketide synthases (PKS), or aminoacyl-AMPs for the peptide and depsipeptide synthetases (Fig. 2). In the PKS complexes the carboxy-activated malonyl-ACP derivative then undergoes decarboxylation, forming a nucleophilic carbanion species that attacks a second acyl thioester to yield a new carbon–carbon bond in one of the steps of polyketide biosynthesis. In peptide and depsipeptide

Figure 1



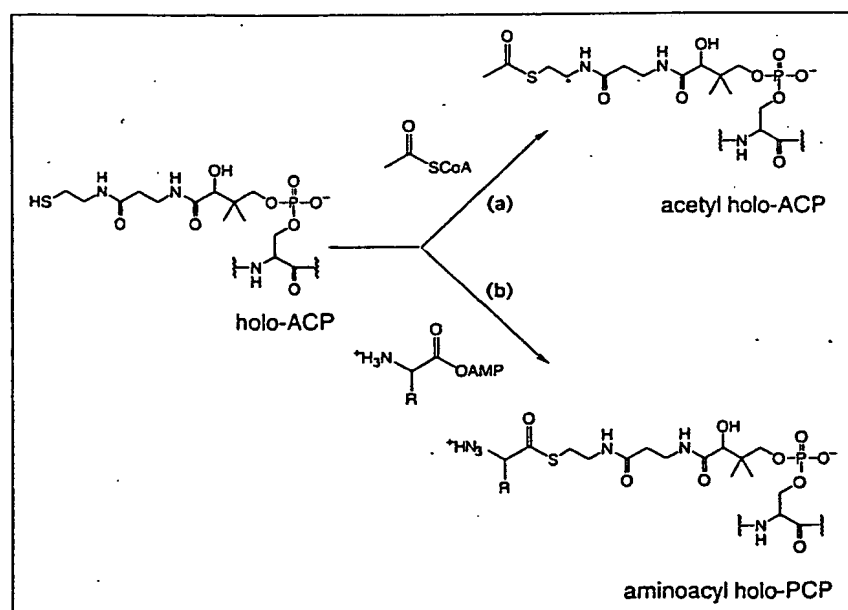
General reaction scheme for posttranslational phosphopantetheinylation. P-pant transferases transfer the 4'-phosphopantetheine moiety

from CoA to a conserved serine residue of apo-ACP to produce holo-ACP and 3',5'-ADP.

synthetases, the aminoacyl-ACPs or hydroxyacyl-ACPs serve as nucleophiles in amide and ester bond-forming steps respectively (Fig. 3). The posttranslational phosphopantetheinylation of apo-ACP domains is clearly essential for the activity of the multienzyme synthetases responsible for the biogenesis of a vast array of natural products. We have therefore searched for and characterized enzymes with P-pant transferase activity. We recently reported the cloning and characterization of the first such transferase, the *Escherichia coli* holo-acyl carrier protein synthase (ACPS), which activates the fatty acid synthase ACP by

converting it to its holo-form [2]. Using the conversion of *E. coli* apo-ACP to holo-ACP as an assay, we purified ACPS 70 000-fold and identified it as the product of a previously described essential *E. coli* gene of unknown function, *dpj* [3]. The *E. coli* ACPS is a 28 kDa dimer of two 125-aa subunits with a k_{cat} of 80–100 min⁻¹ and a $K_M \leq 10^{-6}$ M for apo-ACP. We subsequently showed that the *E. coli* ACPS will also modify apo-forms of several type II ACP homologs including the *Lactobacillus casei* D-alanyl carrier protein (DCP) involved in D-alanylation of lipoteichoic acid [4], the *Rhizobia* protein, NodF, involved

Figure 2



The terminal cysteamine thiol of the phosphopantetheine cofactor acts as a nucleophile for acyl activation. (a) Fatty acid synthases and polyketide synthases transfer acyl groups from acyl-CoA to the phosphopantetheine tether attached to ACP. (b) Non-ribosomal peptide and depsipeptide synthetases first activate their amino-acyl or acyl substrates as their acyl-adenylates before transfer to the phosphopantetheine tether of PCP.

in the acylation of the oligosaccharide-based nodulation factors [5], and the *Streptomyces* ACPs involved in frenolicin, granaticin, oxytetracycline, and tetracenomycin polyketide antibiotic biosynthesis (AMG, RHL and CTW, unpublished results).

The *E. coli* ACPS does not detectably transfer P-pant to the apo-forms of two type I P-pant-requiring proteins involved in amino acid activation, namely apo-EntF which is involved in L-serine activation during *E. coli* enterobactin biosynthesis [6,7] and apo-PCP, a peptidyl carrier protein fragment from the *Bacillus brevis* tyrocidine synthetase (TycA) [8]. Thus other P-pant transferases, specific for the apo-forms of type I peptide synthetases, must exist. Our search in the completely sequenced *Haemophilus influenzae* [9] and *Saccharomyces cerevisiae* genomes for functional homologs of *E. coli* *acpS* initially failed to reveal genes with any apparent homology despite the fact that posttranslational phosphopantetheinylation of ACP domains clearly occurs in these organisms. We report here that more refined database searches yielding peptide sequences with only marginal similarity to ACPS, have in fact led us to identify a large second family of P-pant transferases including the *E. coli* EntD and *B. subtilis* Sfp proteins. The genes encoding these proteins have previously been shown to be required for the production of the non-ribosomal peptides enterobactin and surfactin, respectively (Fig. 4) [10,11]. Putative P-pant transferases have also been identified in *H. influenzae* and *S. cerevisiae* (Fig. 5 and Table 1). We have overproduced and purified EntD, Sfp and a third

E. coli protein o195 and have demonstrated the ability of each to catalyze the transfer of 4'-phosphopantetheine from CoASH to apo-protein substrates.

Results

Database search for ACP synthase homologs

BLAST searches (basic local alignment search tool) [12] with the 125-aa *E. coli* ACPS protein sequence revealed marginal similarity to the carboxy-terminal region of five fungal fatty acid synthases, suggesting that phosphopantetheinylation activity may have been subsumed as a domain in these polyenzymes (Fig. 5). We propose a scheme, based on several lines of genetic evidence [13–18], in which the carboxyl-terminus of the FAS2 subunit could be responsible for the autophosphopantetheinylation of the amino-terminal ACP domain. However, to date we have been unable to demonstrate P-pant transfer from CoASH to the *S. cerevisiae* FAS2 ACP domain (residues Asp142–Ser230) catalyzed by the putative P-pant transferase domain (residues Gly1774–Lys1894) (data not shown).

Using the small similarity between the fungal FAS2 carboxyl-termini and ACPS as a starting point, we detected potential homology to three bacterial proteins, EntD (*E. coli*), Sfp (*B. subtilis*), and Gsp (*B. brevis*) which have previously been identified as genes that appear to have a common ancestor (orthologous genes) (Fig. 5) [19]. Indeed *E. coli* *entD* and *Bacillus brevis* *gsp* can complement *sfp* mutants, supporting the idea that these three proteins have similar functions [19,20]. The specific biochemical

Figure 3

Acyl-pantetheinyl thioesters have a wide variety of fates in the biosynthesis of complex natural products. Acyl-pantetheinyl thioesters can act as (a) carbanion nucleophiles for carbon skeleton assembly in fatty acid and polyketide biosynthesis or as (b) nitrogen or (c) oxygen nucleophiles to yield amide or ester bonds in peptide and depsipeptide biosynthesis.

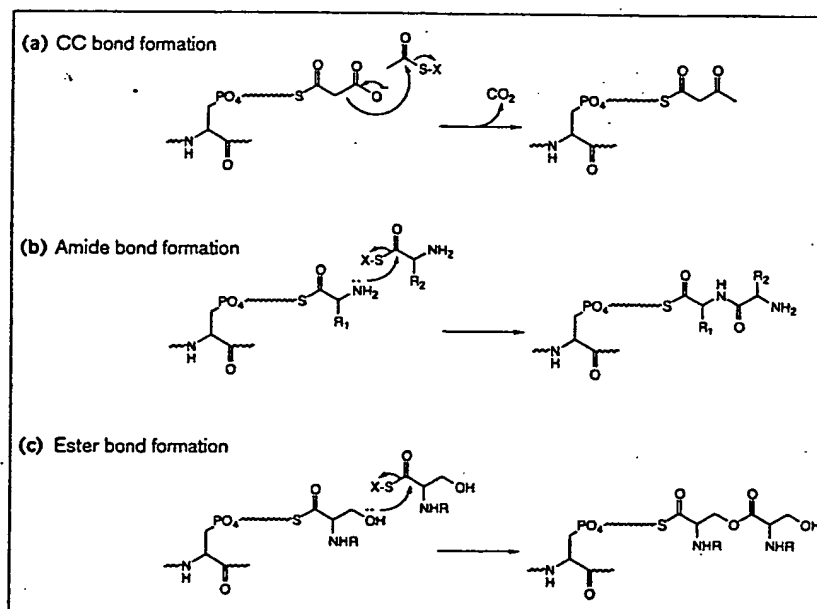
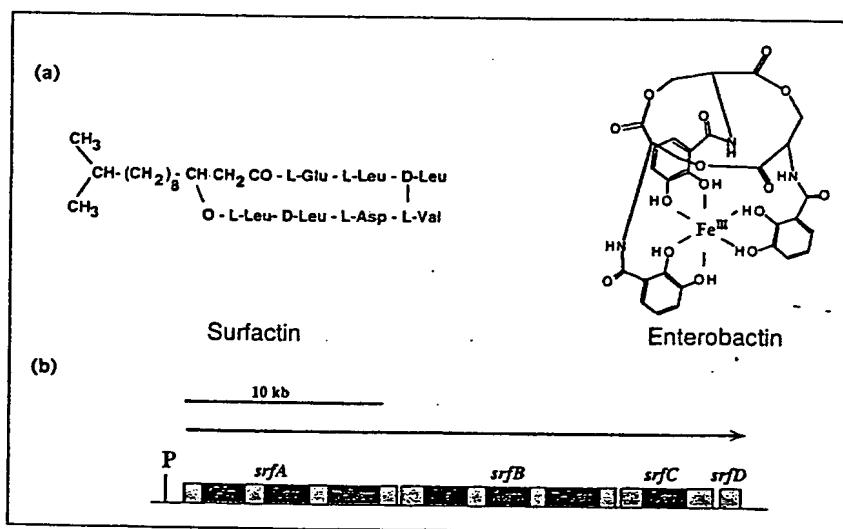


Figure 4



Non-ribosomal peptides and some of the genes involved in their synthesis.

(a) Chemical structures of surfactin and enterobactin. (b) The *srf* operon consists of four open reading frames in which *srfA*, *srfB*, and *srfC* encode for the activities that activate and assemble the seven component amino acids and branched chain β -hydroxy fatty acid of surfactin.

functions of *entD*, *sfp* and *gsp* have up to now remained obscure. *Sfp* was isolated as a locus required for production of the lipopeptide antibiotic surfactin in *B. subtilis* (Fig. 4) [11] and *gsp* is similarly required for gramicidin biosynthesis [19]. Likewise, *entD* has been shown to be required for production of the Fe^{III} -chelating siderophore enterobactin in *E. coli* [10]. Further BLAST searches revealed several other proteins that share potential homology with ACPS (Table 1), including a third *E. coli* open reading frame (in addition to ACPS and EntD) of unknown function designated o195 and proteins involved in cyanobacterial heterocyst differentiation and fungal lysine biosynthesis. Local sequence alignments of the putative P-pant transferase domains reveal two sequence motifs containing several highly conserved residues (Fig. 5, highlighted in yellow).

Confirmation of sequence-predicted P-pant transferase activity

To test the sequence-predicted P-pant transferase activity of this enzyme family, we needed to overproduce and purify representative members of this family (EntD, Sfp and o195), prepare apo-forms of putative substrate proteins or subdomains (ACP, PCP, EntF, and SrfB1) and assay the catalytic competence of the putative enzymes.

Overproduction, purification and characterization of enzymes

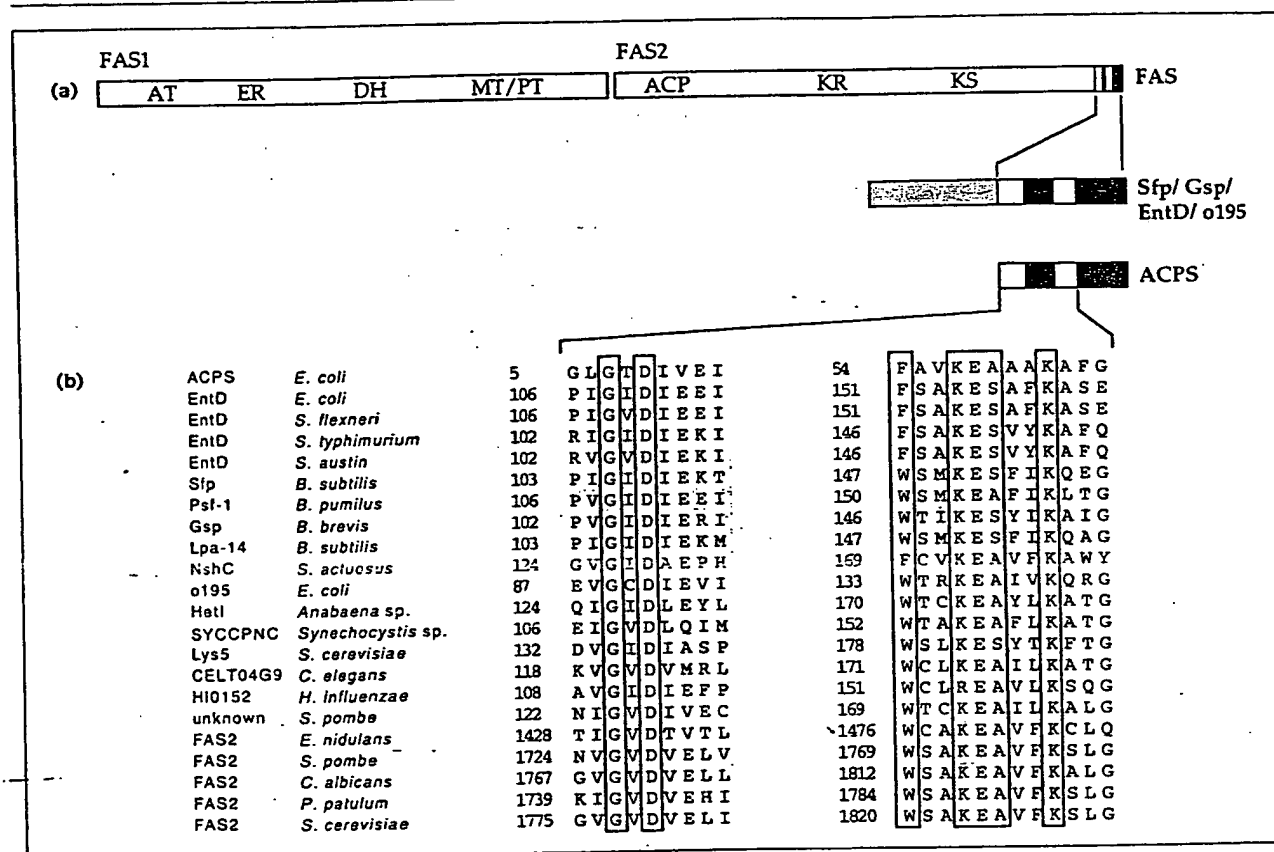
Sfp (26.1 kDa) was overproduced and purified using previously published procedures (Fig. 6) [11]. EntD (23.6 kDa) had previously been cloned, but its overproduction had proven difficult, presumably due to the frequency of rare codons and an unusual UUG start codon [10]. We therefore changed the UUG start to AUG and optimized the codon usage for the first six residues. The *entD* gene

was PCR-amplified from wild type *E. coli* and cloned into the T7-promoter-based pET28b expression plasmid (Novagen). Induction at 25°C yielded soluble EntD, which was purified by ammonium sulfate precipitation and Sephacryl S-100 chromatography. Similarly, the o195 gene was PCR-amplified from wild type *E. coli* cells with codon optimization and cloned into pET28b. Induction at 37°C or 25°C yielded predominantly insoluble o195 protein (21.8 kDa), that could be solubilized in 8 M urea, purified by Q-Sepharose chromatography under denaturing conditions, and renatured by dialysis.

Overproduction, purification and characterization of substrates

Apo-ACP and apo-EntF were overproduced and purified as previously described [7] [21]. Apo-PCP (the peptidyl carrier protein of tyrocidine synthetase, see Fig. 7) and apo-SrfB1 (the first amino acid activation and peptidyl carrier protein domains of surfactin synthetase subunit B) were overproduced in *E. coli* and purified as hexahistidine-tagged proteins using nickel chelate chromatography. Typically, when P-pant-requiring enzymes are over-produced in *E. coli* the fraction of recombinant protein that is modified to the holo-form represents only a small percentage of the total recombinant protein [22]. We have been able to confirm that the percentage of holo-ACP present in the purified preparation is below 5% by using analytical HPLC to resolve the apo and holo-forms of the protein (data not shown) [23]. The ratio of apo- to holo-forms of the other substrates after purification was not precisely determined. It is clear, however, as shown below, that sufficient quantities of the apo-forms of each of these proteins were obtained to act as substrates of the P-pant transferase enzymes. P-pant transferase activity toward each of these substrates was assayed by monitoring

Figure 5



The putative phosphopantetheinyl transferase family. (a) Schematic showing location of the proposed P-pant transferase domains (purple) and location of consensus sequences (yellow) in the fungal fatty acid synthases (FAS), the Sfp/Gsp/EntD/o195 homology family, and *E. coli* ACPS. Component FAS activities are abbreviated as AT, acyl

transferase; ER, enoyl reductase; DH, dehydratase; MT/PT, malonyl/palmitoyl transferase; ACP, acyl carrier protein; KR, ketoreductase; KS, ketosynthase. (b) Local DNA sequence alignments of the consensus sequences of the P-pant transferase enzyme superfamily. Highly conserved residues are boxed.

the transfer of [^3H]-4'-phosphopantetheine from [^3H]- (pantetheinyl)-CoASH in the presence of the putative P-pant transferase enzyme. Reactions were quenched with 10 % trichloroacetic acid (TCA), and the resulting protein pellet was washed, resolubilized, and counted by liquid scintillation to determine the extent to which the apo-substrate was modified to the holo-form by the covalent attachment of [^3H]-4'-phosphopantetheine.

Enzymatic activity with apo-ACP and apo-PCP as substrates
We were initially concerned that large proteins such as EntF (140 kDa) and SrfB (400 kDa) would be difficult to work with as substrates for the preliminary characterization of the putative P-pant transferases. Indeed our prior attempts to modify purified EntF with ACPS had been unsuccessful (RHL, RSF and CTW, unpublished results). Studies with the large, multifunctional chicken fatty acid synthase had shown that, following partial proteolytic digestion, functional domains representative of

component synthase activities could be isolated [24–28]. Indeed, a functional ACP domain of the rat fatty acid synthase had previously been isolated in this manner (S Smith and VS Rangan, personal communication). By identifying the sequence limits of a peptidyl carrier protein (PCP) domain of tyrocidine synthetase (TycA), Marahiel and coworkers have been able to overproduce a functional 112-aa peptide synthetase carrier protein [8] (Fig. 7). This protein undergoes partial phosphopantetheinylation in *E. coli*, and can then act as an aminoacyl acceptor when incubated with its corresponding adenylation/transferase domain. The PCP substrate is easily purified from endogenous *E. coli* ACP when expressed as a hexahistidine fusion (data not shown). An analogous strategy led to construction and isolation of a hexahistidine fusion of SrfB1, a 143 kDa fragment containing the amino-acid-activating and PCP domains involved in the activation of the fourth residue (valine) in surfactin biosynthesis (Fig. 7).

Table 1

ACP synthase homologs.*

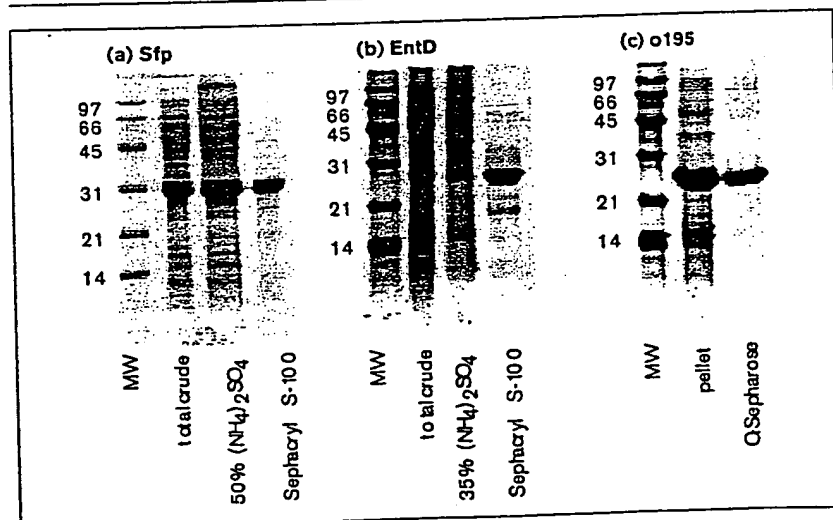
Pathway	Protein	Organism	Size
Enterobactin	EntD	<i>E. coli</i>	209 aa
		<i>S. typhimurium</i>	232 aa
		<i>S. austini</i>	232 aa
		<i>S. flexneri</i>	209 aa
		<i>B. subtilis</i>	224 aa
Surfactin	Sfp	<i>B. pumilus</i>	233 aa
		<i>B. brevis</i>	237 aa
Gramicidin S	Gsp	<i>B. licheniformis</i>	225 aa
Bacitracin	Bli	<i>B. subtilis</i>	224 aa
Iturin A	Lpa-14	<i>S. actuosus</i>	253 aa
Nosiheptide	NshC	<i>S. cerevisiae</i>	272 aa
Lysine	LYS5	<i>E. coli</i>	126 aa
Fatty acids	ACPS	<i>H. influenzae</i>	235 aa
		<i>S. cerevisiae</i>	1894 aa
		<i>C. albicans</i>	1885 aa
		<i>P. patulum</i>	1857 aa
		<i>S. pombe</i>	1842 aa
Differentiation	HetI	<i>A. nidulans</i>	1559 aa
		<i>Anabaena sp.</i>	237 aa
		<i>Synechocystis sp.</i>	246 aa
		<i>E. coli</i>	195 aa
		<i>S. pombe</i>	258 aa
Unknown	o195	<i>C. elegans</i>	297 aa

*All sequences except NshC (W Strohl, personal communication, GenBank Accession Number U75434, submitted) and Bli (M Marahiel, unpublished) are available in the GenBank, SwissProt, or EMBL databases.

As mentioned above, recombinant PCP undergoes partial phosphopantetheinylation when expressed in *E. coli* [8]. When recombinant PCP was incubated with purified ACPS and [^3H](pantetheinyl)-CoASH *in vitro*, however, no incorporation of ^3H label was observed (Fig. 8). This result agreed with our earlier finding that ACPS cannot

catalyze the modification of EntF, another type I peptide synthetase component. We therefore hypothesized that another *E. coli* P-pant transferase activity, probably EntD given its sequence similarity to ACPS, is specific for the phosphopantetheinylation of EntF or recombinant PCP overproduced in *E. coli*. To test this idea, we incubated each of the four pure proteins ACPS, EntD, o195, and Sfp with apo-ACP and apo-PCP in the presence of [^3H]CoASH. Each of the four candidate P-pant transferases generated tritiated ACP and/or PCP in TCA precipitation assays (data not shown). To verify that the ^3H label that coprecipitated with ACP and PCP represented covalent attachment of P-pant, the tritiated products were subjected to SDS electrophoresis and autoradiography (Fig. 8). It is clear that both ACPS and Sfp show robust phosphopantetheinylation activity (Fig. 8a). When apo-ACP is the substrate, EntD is weakly active compared to ACPS and Sfp and o195 is even less active, but both EntD and o195 give signals well above the background, showing that EntD and o195 do have P-pant transferase activity. When the 13 kDa apo-PCP was used as substrate for these four P-pant transferases in Figure 8b, Sfp and EntD are now highly active, but o195 and ACPS give no detectable modification at the single timepoint. When the much larger substrates apo-EntF and apo-SrfB1 fragment (140 kD) are used (Fig. 8c), the cognate enzymes, EntD for EntF and Sfp for SrfB1, are obviously competent for posttranslational phosphopantetheinylation. Mass spectrometry was used to confirm that the tritium incorporated into the apo-proteins represented transfer of the intact phosphopantetheinyl group. We previously validated this approach using ACPS as catalyst and holo-ACP as product [2] and used it here to examine PCP modification. Mass spectrometric analysis (MALDI-TOF) of unlabeled enzymatic holo-PCP indicated a molecular

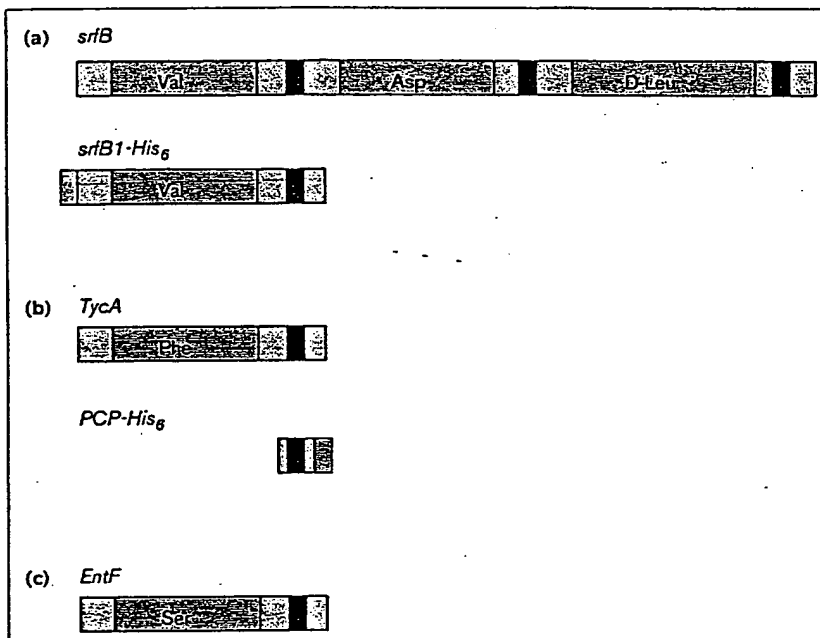
Figure 6



Overproduction of candidate P-pant transferases. (a) Purification of *Bacillus subtilis* Sfp heterologously expressed in *Escherichia coli*. (b) Overproduction and purification of *E. coli* EntD. (c) Overproduction and purification of *E. coli* o195. All gels shown are SDS-PAGE (15 % acrylamide, 2.6 % bisacrylamide).

Figure 7

P-pant acceptor domains and the His₆-tagged constructs used for purification. Schematic diagram showing the comparative alignment of (a) SrfB and the SrfB1-His₆ fragment, (b) TycA and its constituent PCP domain tagged with His₆ and (c) EntF. Amino-acid-activating domains are shown in light purple. Phosphopantetheine attachment sites are shown in dark purple.



weight of 13 431 (calculated 13 459) in contrast to an observed molecular weight of 13 130 (calculated 13 120) for the apo-PCP substrate. These are the first data that establish that EntD, Sfp, and o195 are enzymes and that they catalyze the transfer of P-pant to the serine sidechain of an acyl carrier protein.

Specificity of ACPS, EntD and o195

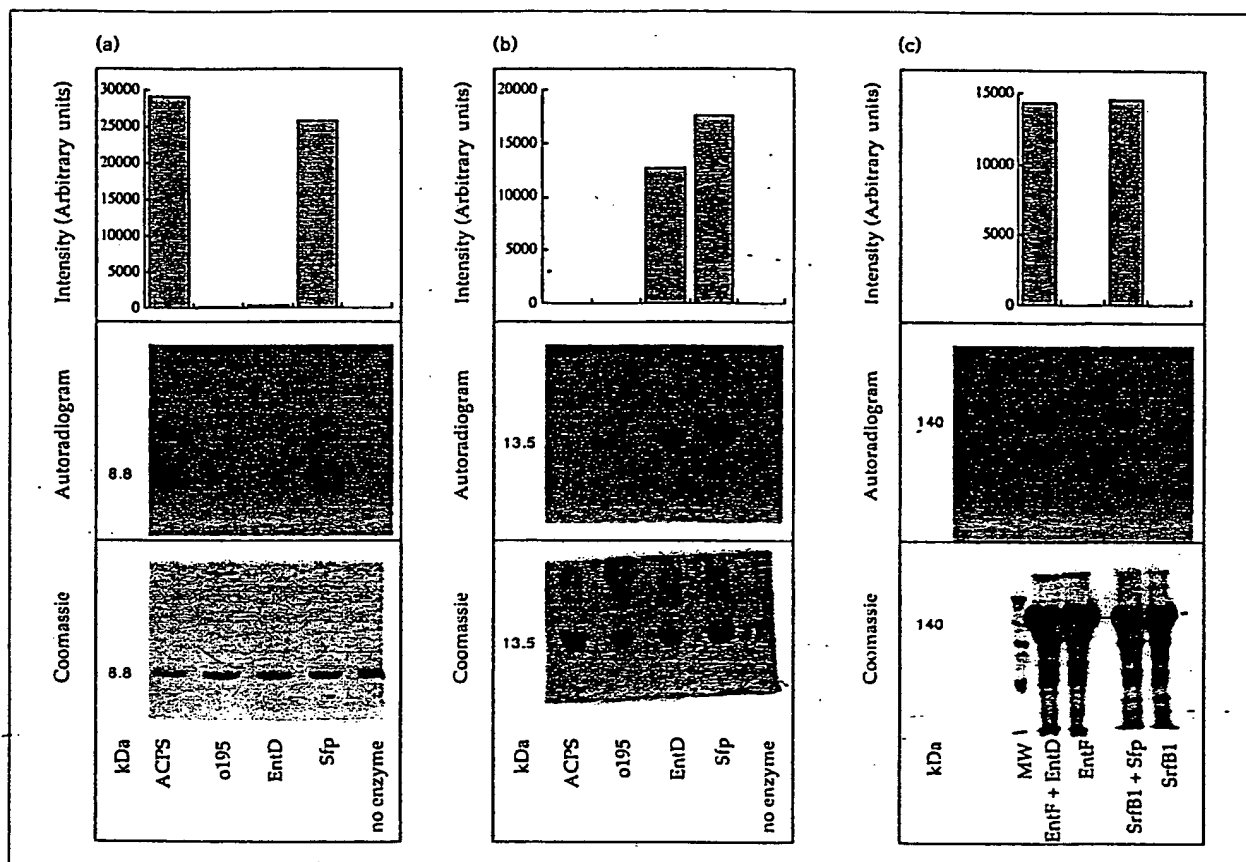
Having demonstrated that EntD does in fact have P-pant transferase activity, we sought kinetic confirmation that it is indeed the enzyme responsible for the posttranslational modification of EntF. As described above, autoradiography of SDS gels confirmed incorporation of radiolabeled phosphopantetheine into EntF catalyzed by EntD (Fig. 8c). Furthermore, a time course of EntD-catalyzed incorporation of radiolabel into EntF provides *in vitro* evidence of at least two partner-specific P-pant transfer reactions occurring within *E. coli*. ACPS specifically catalyzes the transfer of P-pant to apo-ACP, while EntD is the transferase for its partner EntF. EntF is modified effectively by EntD (100 nM), whereas EntF undergoes almost no modification in the presence of 15-fold higher concentrations of ACPS and o195, clearly demonstrating the specificity of EntD for EntF (Fig. 9a). In contrast, apo-ACP is almost exclusively modified by ACPS (Fig. 9b), confirming that in *E. coli* ACPS is the P-pant transferase that activates the type II fatty acid synthase and EntD is the P-pant transferase that activates the type I enterobactin synthetase. The autoradiogram in Figure 8a shows, however, that both o195 and EntD can

modify apo-ACP; the rate of modification is very low, yet is significantly higher than the background rate in the absence of enzyme (Fig. 8a, lane 5). This is presumably due to non-specific enzyme-catalyzed phosphopantetheinylation of the conserved serine residue. Assuming that the inclusion-bound o195 has been properly refolded and that an additional glycine introduced after the methionine start during PCR cloning has no significant effect on activity, it would appear that o195 is specific for a third, as yet unknown, substrate in *E. coli*; presumably P-pant transfer to this unknown protein would require o195 and would not be efficiently catalyzed by ACPS or EntD.

Specificity of Sfp toward apo-SrfB1, apo-PCP and apo-ACP

Sfp appears to be non-specific, efficiently catalyzing the modification of the two *Bacillus* derived type I peptide synthetase domains, apo-PCP and apo-SrfB1, the *E. coli* type II fatty acid synthase apo-ACP subunit (Fig. 8) and EntF (data not shown). Based on this evidence, Sfp would appear not to discriminate between type I peptide synthetase domains and type II fatty acid synthase subunits suggesting that there may be crosstalk between Sfp and fatty acid synthase, at least when expressed in *E. coli*. Careful kinetic analysis to determine whether Sfp selectively modifies SrfABC and not the *B. subtilis* fatty acid synthase ACP subunit must await overproduction of the *B. subtilis* ACP, however. Morbidino and co-workers [29] have been able to sequence the entire *B. subtilis* ACP protein by Edman degradation, but the intact *acpP* gene appears to be toxic to *E. coli* and has proven difficult to clone.

Figure 8



P-pant transferase reactions. Coomassie-stained gels are shown for each P-pant transferase incubation with the corresponding autoradiograms and integrated band intensities for individual P-pant transferase incubations. (a) Incubations of ACPS (1.8 μ M), o195 (2.2 μ M), EntD (1.3 μ M), Sfp (1.6 μ M) or no enzyme with apo-ACP

(150 μ M) as substrate. (b) Incubations of ACPS (1.8 μ M), o195 (2.2 μ M), EntD (1.3 μ M), Sfp (1.6 μ M) or no enzyme with apo-PCP (45 μ M) as substrate. (c) Incubations of EntD (1.3 μ M) and Sfp (1.6 μ M) with their homologous substrates apo-EntF and apo-SrfB1.

Holo-SrfB1 can activate L-valine

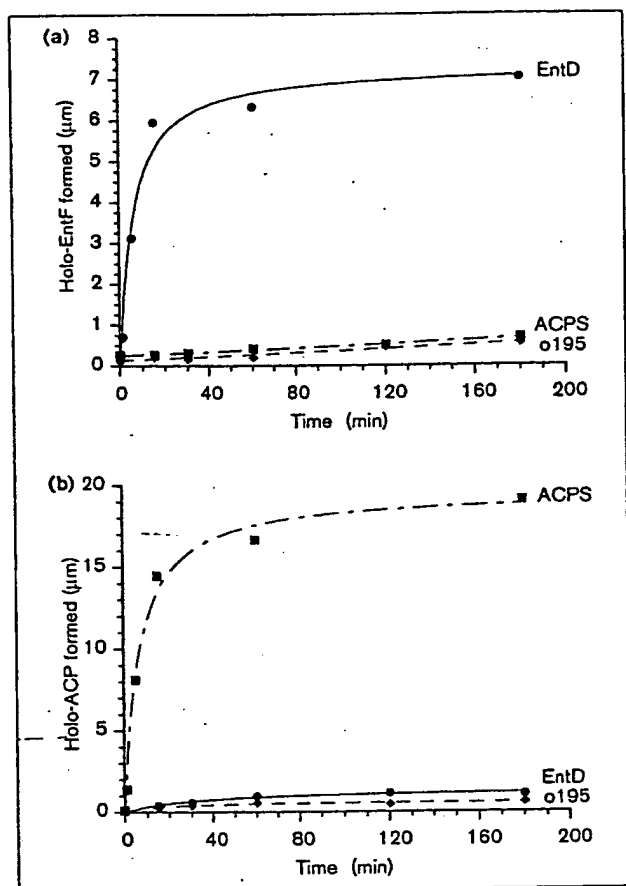
The action of Sfp on the 143 kDa SrfB1 fragment in conversion of the apo-form to the holo-form (Fig. 1) should generate a phosphopantetheinylated SrfB1 competent to undergo specific recognition and acylation by the amino acid L-valine, residue 4 in surfactin (Figs 4,7). Apo-SrfB1 undergoes very little acylation when incubated with [14 C]-L-valine, showing that the contamination of this preparation by holo-SrfB1 is small. After incubation with Sfp, however, the level of [14 C]-L-valine-holo-SrfB1 covalent complex formed in the complete incubation mixture increases about 14-fold, consistent with an increase in the amount of holo-SrfB1 present. The [14 C]-L-valine is used by the amino-acid-activating domain of holo-SrfB1 to make valyl-AMP which then undergoes intramolecular acyl-transfer to the SH group of the P-pant moiety in the adjacent PCP domain. Holo-SrfB1 cannot be covalently acylated by the non-cognate L-aspartate residue, the fifth

amino acid to be activated by SrfABC, as expected given the absence of an aspartate-specific adenylation domain on SrfB1. Thus the holo-SrfB1 formed following incubation with Sfp and CoASH has both an active adenylation domain and a functional holo-peptidyl carrier protein domain, and should therefore be a useful reagent to probe peptide-bond-forming steps between adjacent sites of multienzyme, multiple thiotemplate synthases.

Discussion

The transfer of 4'-phosphopantetheine from CoASH to conserved serine residues in the signature sequences of acyl carrier protein domains (type I) or subunits (type II) is essential for the functional activation of all fatty acid synthases, polyketide synthases and non-ribosomal peptide synthetase complexes. This posttranslational phosphopantetheinylation introduces a covalently-attached

Figure 9



Time courses of P-pant transferase activity. (a) Time course of EntD (100 nM), ACPS (1.8 μ M), or o195 (1.5 μ M) incubated with apo-EntF (20 μ M) as measured by radioassay. (b) Time course of EntD (1.6 μ M), ACPS (100 nM), or o195 (1.5 μ M) incubated with apo-ACP (50 μ M).

nucleophilic thiol on a long tether that becomes the site of all the initiation and acyl transfer events involved in the assembly of the broad array of natural products synthesized by these enzymes. Thus, identification of the P-pant loading enzymes that create the active holo-ACP forms by posttranslational modification is important to the understanding of both the molecular mechanism of holo-ACP formation and the specificity of serine phosphopantetheinylation. These findings will aid in the design of strategies for heterologous production of functional polyketide and polypeptide synthetases (e.g. in combinatorial biosynthesis of 'unnatural' natural products), and studies aimed at the synthesis of inhibitors of specific P-pant loading reactions (e.g. in fungal lysine biosynthesis, see below).

Our recent purification, characterization, and identification of the *E. coli* holo-ACPS [2] provided the first

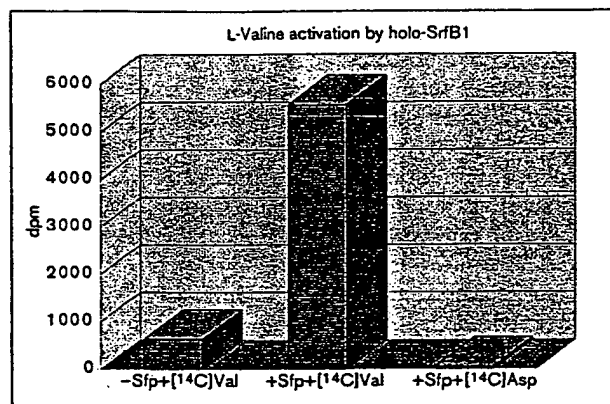
molecular information on this class of posttranslational-modifying enzymes. Somewhat to our surprise, initial database searches with the *E. coli* ACPS sequence revealed no obvious homologs in the protein databases. We eventually detected marginal similarities of 15–22 % over 120 residues in the carboxy-terminal region of three fungal fatty acid synthases (Fig. 5), indicating that the phosphopantetheinylating activity may have been integrated as a domain in these polyenzymes. For example the carboxy-terminal 121 aa of the 1894-aa yeast fatty acid synthase subunit II (γ FASII) might act intramolecularly to add a P-pant unit to Ser180 on the putative ACP domain of this polyprotein. We have not yet obtained active fragments of γ FASII that catalyze these reactions *in trans*, but Schweizer's group [13–18] has previously reported that two mutated fatty acid synthases, one in which the mutation is at Ser180 and the other at Gly1777, which are inactive alone, can complement each other *in vivo* and *in vitro*, consistent with this proposal.

EntD, Sfp and Gsp as specific P-pant transferases

Starting with *E. coli* ACPS, we detected three bacterial proteins EntD, Sfp, and Gsp which have previously been identified by complementation as orthologous genes [19,20]. The specific functions of *sfp*, *gsp* and *entD* have until now been obscure. The studies described here establish that Sfp has phosphopantetheinyl transferase activity and clearly assigns a catalytic loading function to Sfp. It posttranslationally modifies the conserved serine in the first subsite of SrfB, which is responsible for valine activation. We expect that Sfp will be able to modify the consensus serine residue in all seven amino-acid-activating sites in SrfABC (Fig. 4) and by extension that Gsp will catalyze P-pant transfer to the five amino-acid activating sites in GrsA and GrsB, allowing the sequential activation and polymerization of amino acids as required for the ribotemplate mechanism for non-ribosomal peptide bond assembly [30]. The *bli* and *lpa-14* gene products most probably have an equivalent role, that is iterative P-pantetheinylation of each amino acid-activating domain in *B. licheniformis* bacitracin synthetase [31] and *B. subtilis* iturin A synthetase respectively [32]. While *in vitro* enzymatic specificity remains to be fully explored, the *in vivo* genetic studies [11,32] argue strongly for specific partner protein recognition by a distinct P-pant transferase. This may well be a general theme in non-ribosomal peptide antibiotic biosynthesis. While Sfp, Gsp and EntD are required for peptide and depsipeptide biosynthesis, these proteins are not essential for survival [10,33]. We predict, however, that there will be other as yet unidentified P-pant transferases in the *Bacillus* organisms specific for the ACP subunits of their respective fatty acid synthases which, like *E. coli* ACPS, will be essential for viability.

A third example of a partner protein-specific phosphopantetheinyl transferase is EntD, one of the proteins

Figure 10



[¹⁴C]Valine activation by holo-SrfB1. In the first column, SrfB1 (2 μ M) was preincubated with CoA (200 μ M) in the absence of Sfp before subsequent incubation with [¹⁴C]-L-Valine (100 μ M, 42.4 Ci mol⁻¹) and ATP (2 mM). In the second column, SrfB1 was preincubated with CoA (200 μ M) in the presence of Sfp (1.3 μ M) before subsequent incubation with [¹⁴C]-L-Valine (100 μ M, 42.4 Ci mol⁻¹) and ATP (2 mM). In the third column, SrfB1 (2 μ M) was preincubated with CoA (200 μ M) in the presence of Sfp (1.3 μ M) before subsequent incubation with [¹⁴C]-L-Aspartate (100 μ M, 40.3 Ci mol⁻¹) and ATP (2 mM).

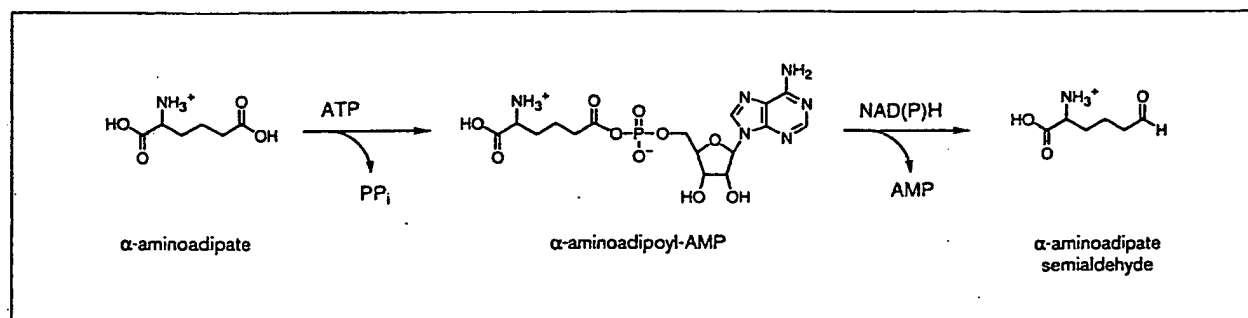
—required for production and secretion of the iron-scavenging dihydroxybenzoyl-serine trilactone enterobactin in *E. coli*. We had previously cloned, sequenced, and purified EntF, a 140 kDa component of the enterobactin synthetase, and demonstrated that it activates L-serine and contains phosphopantetheine [6,7]. As EntD is required for enterobactin biosynthesis *in vivo* [10] and shows high activity for *in vitro* P-pantetheinylation of pure apo-EntF, it is now clear that EntD is defined as the specific P-pant transferase that makes active holo-EntF from apo-EntF *in vivo*. Pure ACPS from *E. coli* will not significantly posttranslationally modify EntF, consistent with the hypothesis that protein-protein recognition

controls the specificity of phosphopantetheinylation *in vivo*. We predict that incubations of EntD and the enterobactin synthetase components with CoASH, L-serine and dihydroxybenzoate should reconstitute *in vitro* enterobactin production. At 140 kDa, EntF is the appropriate size for an amino-acid-activating module in a multidomain polypeptide synthetase [34]. It can be efficiently modified *in vitro* by EntD, showing that P-pant addition can occur after translation of the apo-protein, and not only co-translationally prior to folding of the apo-protein into its native structure. The NMR structure of *E. coli* apo-ACP shows that the nucleophilic Ser36 is in an accessible β -turn [35]; this may be a common architectural scaffolding for ACP domains in polyketide and polypeptide synthetases and may be important in recognition by P-pant transferases.

Other P-pantetheinyl transferases

Using the EntD/Sfp/Gsp family as a base for further database searches has led to the identification of several additional candidates that are probably P-pant transferase family members (Table 1). Of these, in addition to ACPS and EntD, we have subcloned, expressed and characterized o195 as a third *E. coli* protein with P-pant transferase activity. The activity of o195 towards apo-ACP and apo-EntF is low, suggesting that o195 specifically catalyzes efficient P-pant transfer to an as yet unidentified substrate. A hypothetical protein, HI0152, in *Haemophilis influenzae* has been identified as a putative P-pant transferase. This resolves the apparent problem that no P-pant transferase in the *Haemophilis* genome had previously been found using ACPS-based searches. HI0152 is positioned directly upstream of the *H. influenzae* fatty acid synthase gene cluster, consistent with the notion that its protein product might be involved in fatty acid biogenesis. There is also some evidence that two additional proteins in cyanobacteria have similar functions (Table 1). In *Anabaena*, the genes *HetI*, *HetM*, and *HetN* have been implicated in the production of an unidentified secondary metabolite that inhibits heterocyst differentiation (a

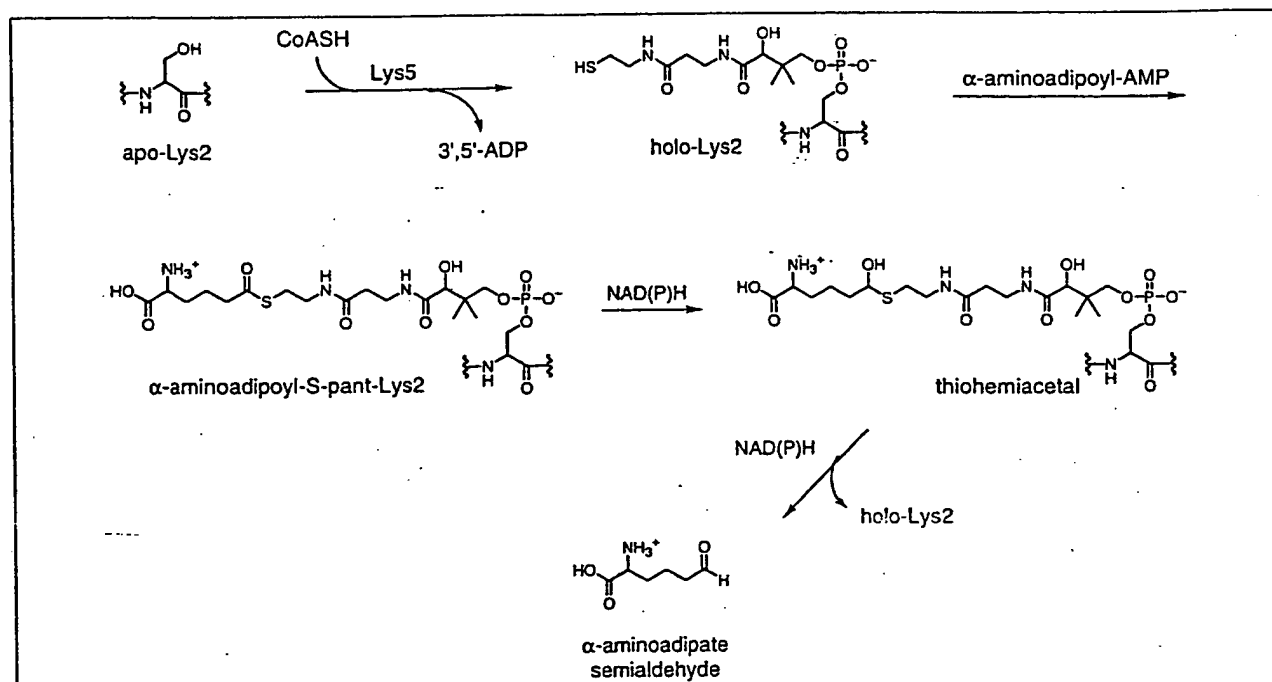
Figure 11



Scheme showing the reaction previously proposed to be catalyzed by the Lys2-Lys5 complex. α -Aminoadipate is first activated to α -amino-

adipoyl-AMP. This acyl-adenylate would then undergo direct reduction in a NAD(P)H dependent reaction to yield α -aminoadipate semialdehyde.

Figure 12



Scheme showing the reaction we now propose to be catalyzed by Lys2. Following phosphopantetheinylation of Lys2 catalyzed by Lys5, aminoadipate is transferred from aminoadipoyl-AMP to yield α -amino-

adipoyl-S-pant-Lys2. This thioester then undergoes direct reduction in a NAD(P)H dependent reaction to yield a thiohemiacetal intermediate which then decomposes to the α -aminoadipate semialdehyde.

process occurring under low fixed nitrogen conditions in which a subset of cyanobacterial cells differentiate into the specialized heterocysts which have the ability to fix nitrogen [36]. Sequence analysis suggests HetN is a NAD(P)H-dependent oxidoreductase like those involved in the biosynthesis of polyketides and fatty acids, while HetM has an ACP domain. HetI shows similarity to Sfp/Gsp/EntD, and is thus likely to be the HetM-specific phosphopantetheinyl transferase in the synthesis of the hypothesized secondary metabolite.

A final example is the 272-aa Lys5 protein involved in the yeast lysine biosynthetic pathway. Yeast and other fungi synthesize lysine via the unique α -aminoadipate pathway, an eight-step pathway beginning with homocitrate and proceeding via α -aminoadipate to saccharopine to lysine [37]. Complementation analysis suggests that Lys2 and Lys5 are involved in the same step in this pathway, the reduction of α -aminoadipate to aminoadipate semialdehyde [38]. Labeled pyrophosphate exchange experiments indicate that this reaction appears to proceed through an α -aminoadipoyl-AMP intermediate [39,40]. Recent sequence analysis [41] shows Lys2 to be a 155 kDa protein with homology to amino-acid-activating peptide synthetases including TycA, GrsAB, and SrfA. Like these peptide synthetases, Lys2 is believed to cleave

ATP to AMP and PPi, activating α -aminoadipate to the α -aminoadipoyl-AMP which is then reduced by NADPH to the aldehyde (Fig. 11). A search for a consensus P-pant attachment site in Lys2 reveals the signature motif LGGHS around Ser880. We therefore propose, in contrast to previous suggestions, that Lys2 and Lys5 may form a two-subunit enzyme [38], that the 272-aa Lys5 is a specific phosphopantetheinyl transferase for Ser880 in Lys2. The thiol of the newly-introduced P-pant prosthetic group on Lys2 would attack the aminoadipoyl-AMP to give aminoadipoyl-S-pant-Lys2, in a similar manner to the sequential formation of aminoacyl-AMP to aminoacyl-S-pant-TycA in the homologous tyrocydine synthetase A subunit (Fig. 12). At this point, hydride addition to the acyl-S-pant-Lys2 would yield a thiohemiacetal which would readily decompose to aldehyde product and HS-pant-Lys2. This sequence has precedent in the reverse direction in the oxidation of glyceraldehyde-3-P to the acyl-S-enzyme in GAP dehydrogenase catalysis via a cysteinyl-S-enzyme hemithioacetal [42].

Significance

We have obtained evidence for a family of more than a dozen proteins with catalytic posttranslational modification activity. We anticipate that all these proteins will prove to be phosphopantetheinyl transferases with

CoASH as a common substrate but will show specificity, directed by protein-protein interactions, for the conserved serine motif in particular partner proteins. It is likely that most, if not all, of the multienzyme peptide synthetases that use the multiple thiotemplate scaffolding strategy to make peptide antibiotics nonribosomally [30] will have a partner-protein-specific posttranslational modifying enzyme that covalently adds the swinging arm thiol group required to enable acyl transfers. The new proteins in this family are 50–150 amino acid residues longer than the first one discovered, the 125-aa *E. coli* ACPS subunit; these extra amino acids may be responsible for specificity of partner-protein binding. It remains to be seen whether the many polyketide synthase complexes will use this strategy for posttranslational modification.

Materials and methods

Overproduction, purification and characterization of EntD, Sfp, and $\alpha 195$

B. subtilis Sfp was overproduced and purified from *E. coli* strain MV1190/pUC8-sfp as previously described by Nakano *et al.* [11] (Fig. 6). EntD was PCR-amplified from wild-type *E. coli* K-12 by colony PCR using the forward primer 5'-ATTATATCCATGGGTCCTCCGTTC-CAACATGGTCGATATGAAACTACGCA-3' and the reverse primer 5'-TGATGTCAAAGCTTATTAATCGTGTGGCACAGCGTTAT-3' (IDT). The forward primer introduced an *NcoI* restriction site (underlined) which allowed mutation of the TTG start to an ATG start and inserted a Gly codon (GGT) after the Met initiator. In addition the forward primer optimized codon usage for the first six codons of the *entD* gene (modified bases shown in lower case). The reverse primer incorporated a *HindIII* restriction site (underlined). The *NcoI/HindIII* digested PCR product was cloned into pET28b (Novagen) and transformed into competent *E. coli* DH5 α . The recombinant *entD* sequence was confirmed by DNA sequencing (Dana-Farber Molecular Biology Core Facility, Boston, MA). Competent cells of the overproducer strain *E. coli* BL21(DE3) were then transformed with the supercoiled pET28b-*entD*. Induction of a 2-l culture of BL21(DE3)pET28b-*entD* with 1 mM isopropyl- β -D-thiogalactopyranoside (IPTG) followed by growth at 25°C for 5 h yielded predominantly inclusion-bound EntD, although a modest amount of the overproduced protein was soluble. The overproduction of soluble EntD may be complicated by the fact that the wild type Ent proteins are synthesized in detectable quantities only under iron-starved conditions. Furthermore, although the recombinant EntD is functional as a soluble protein, the wild type EntD has been reported to be membrane bound [43]. The induced cell paste was resuspended in 50 mM Tris, 1 mM EDTA, 5 % glycerol, pH 8.0 (40 ml) and lysed by two passages through the French press at 15 000 psi. Cellular debris and inclusion bound protein was removed by centrifugation at 8000 \times g for 30 min. Pulverized ammonium sulfate was added to 35 %, 65 % and 80 % saturation. The 35 % fraction containing the largest fraction of EntD was applied to a 2.5 \times 115 cm Sephacryl S-100 column. The column was eluted at a flow rate of 1 ml min⁻¹ using the same buffer as above, collecting 8 ml fractions to obtain homogeneous protein.

Similarly, $\alpha 195$ was PCR-amplified from wild-type *E. coli* K-12 by colony PCR using the forward primer 5'-ATTATATCCATGGGTTACCGGATAGTTCTGGGGAAAGTT-3' and the reverse primer 5'-TGATGTCAAAGCTTATCAGTTAACTGAATCGATCCATTG-3' (IDT). The forward primer with its *NcoI* restriction site (underlined) gave insertion of a Gly codon (GGT) after the Met initiator codon of the $\alpha 195$ sequence; codon usage for the succeeding codon was also optimized (base change shown in lower case). The reverse primer incorporated a *HindIII* restriction site (underlined). The *NcoI/HindIII*-digested PCR product was cloned into pET28b (Novagen) and transformed into competent

E. coli DH5 α . The recombinant $\alpha 195$ sequence was confirmed by DNA sequencing (Dana-Farber Molecular Biology Core Facility, Boston, MA). Competent cells of the overproducer strain *E. coli* BL21(DE3) were then transformed with the supercoiled pET28b- $\alpha 195$. Induction of a 2-l culture (2 \times YT media) of BL21(DE3)pET28b- $\alpha 195$ with 1 mM IPTG followed by growth at 37°C for 3.5 h yielded predominantly inclusion-bound $\alpha 195$ protein. The cell paste was resuspended in 50 mM Tris-HCl, 1 mM EDTA, 5 % glycerol, pH 8.0 (40 ml) and lysed by two passages through a French pressure cell at 15 000 psi. Cellular debris and inclusion-bound protein was pelleted by centrifugation at 27 000 \times g for 30 min. The inclusion-bound protein pellet was resuspended in 30 ml of 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 5 % glycerol and incubated for 30 min at room temperature with 10 mg lysozyme and 30 mg deoxycholate. The pellet was reobtained by centrifugation for 15 min at 27 000 \times g and solubilized in 30 ml of 8 M urea, 50 mM Tris-HCl, pH 8.0, 10 mM dithiothreitol (DTT). Residual solid material was removed by centrifugation for 15 min at 27 000 \times g. The urea-solubilized solution (30 ml) was then applied to a 2.5 \times 10 cm Q-Sepharose column equilibrated with 8 M urea, 50 mM Tris-HCl, pH 8.0. The column was washed with 50 ml of the equilibration buffer and then a gradient of 250 ml 0–0.25 M NaCl in 8 M urea, 50 mM Tris-HCl pH 8.0 followed by 200 ml of 0.25–1 M NaCl in the same buffer was applied. The $\alpha 195$ protein eluted at \sim 200 mM NaCl as determined by 15 % SDS-PAGE. The purified $\alpha 195$ was renatured by diluting a portion of it 10-fold in 8 M urea, 50 mM Tris-HCl, pH 8.0, 10 mM DTT and dialyzing overnight at 4°C against 10 mM Tris-HCl, pH 8.0, 1 mM DTT. Two liters of culture grown in 2 \times YT media yielded 3.1 g of cells from which \sim 80 mg of $\alpha 195$ protein was obtained.

Production of apo-protein substrates, apo-ACP, apo-PCP, apo-EntF, and apo-SrfB

The *E. coli* fatty acid synthase ACP was overproduced and purified in its apo-form from *E. coli* strain DK554 [21] following the procedure of Rock and Cronan [44] with the exception that following cell disruption and centrifugation (30 min at 28 000 \times g), the crude extract containing 10 mM MgCl₂ and 10 mM MnCl₂ was incubated for 60 min at room temperature. In this manner, minor amounts of holo-ACP were hydrolyzed to the apo-form using the endogenous *E. coli* ACP phosphodiesterase [45]. The PCP domain of TycA was overproduced with a hexahistidine tag using *E. coli* strain SG13009(pREP4)/pQE60-PCP [8]. Following lysis of the induced culture the His₆-tagged protein was purified by nickel-chelate chromatography. *E. coli* apo-EntF was purified as previously described [7].

Apo-SrfB1 was cloned from plasmid p120-21E [46]. Briefly, p120-21E was digested with EcoRV to release a 3648-base-pair fragment encoding the SrfB1, valine-activating domain of surfactin synthetase. This fragment was inserted into *StuI*-cleaved pPROEX-1 (Gibco/BRL Life Sciences Technologies) to give plasmid pML118 which codes for a amino-terminal His₆-tagged SrfB1 domain (142.7 kDa). His₆-SrfB1 was overproduced using *E. coli* strain AG1574 (courtesy A. Grossman) [47]. Cells were grown at 25°C in 2 \times YT media (2 l) to an O.D. of 0.4 at which point they were induced with 1 mM IPTG and allowed to grow for an additional 4 h. Cells were harvested by centrifugation (3 g), resuspended in 35 ml of 5 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl, pH 7.9 and lysed by two passages through a French pressure cell. This crude extract was clarified by centrifugation for 30 min at 27 000 \times g. More than 50 % of the overproduced SrfB1 was obtained in the soluble fraction as determined by 6 % SDS-PAGE. His₆-tagged SrfB1 was purified on His-Bind resin (Novagen) following the manufacturer's recommendations.

Assay for apo-protein to holo-protein conversion by ³H-P-pant group transfer from ³H-coenzyme A

P-pant transferase activity (Fig. 1) was measured by radioassay. Enzyme preparations (final enzyme concentrations of 0.1–2.2 μ M) were incubated with 75 mM Tris-HCl, pH 8.8, 10 mM MgCl₂, 25 mM DTT, 200 μ M [PH]-(pantetheinyl)-CoASH (5.3 \times 10⁶ dpm total activity)

and substrate (apo-ACP, apo-PCP, apo-EntF or apo-SrfB1, at final concentrations of 10–150 μ M) for various times at 37° C in a final volume of 100 μ l. The incubations were quenched with 10 % TCA and 500 μ g bovine serum albumin (BSA) was added as a carrier. The protein was precipitated by centrifugation, washed 3 times with 10 % TCA, and the protein pellet solubilized with 150 μ l 1 M Tris base. The resuspended protein was added to 3 ml liquid scintillation cocktail and the amount of [3 H]-phosphopantetheine incorporated into the substrate protein was quantified by liquid scintillation counting. Assays for autoradiography were performed as described above except 20 μ M [3 H]-(pantetheinyl)-CoASH (2.6 \times 10⁸ dpm total activity) was used in the assay, no BSA was added to the TCA precipitate, and pellets were solubilized in SDS or native PAGE sample buffer titrated with 1 M Tris base. Assays using apo-PCP as substrate were resolved by 15 % SDS-PAGE, assays using *E. coli* ACP were resolved by 20 % native PAGE, and assays using SrfB1 or EntF were resolved on 8 % SDS-PAGE. Gels were Coomassie-stained, soaked for 30 min in Amplify (Amersham), dried at 80° C under vacuum and exposed to X-ray film for 24–150 h at –70° C (Fig. 8). The autoradiograms were scanned using a digital scanner and relative intensities of the radiolabeled bands were quantified using NIH Image 1.59 software (National Institutes of Health, USA).

Assay for activation of L-valine by holo-SrfB1

Apo-SrfB1 (2 μ M) was incubated with 200 μ M CoASH, 75 mM Tris-HCl pH 8.0, 10 mM MgCl₂, 25 mM DTT and 1.3 μ M Sfp for 15 min at 37° C to generate holo-SrfB1. To the SrfB1-Sfp reaction mixture, ¹⁴C-labeled amino acid (valine, 42.4 Ci mol^{–1}; aspartic acid, 40.3 Ci mol^{–1}) was added to 100 μ M final concentration. ATP was added to a final concentration of 2 mM and the reaction (115 μ l) was incubated for 15 min at 37° C, then stopped by the addition of 800 μ l 10 % TCA with 15 μ l of a 25 mg ml^{–1} BSA solution as carrier. The precipitate was collected by centrifugation, washed with 10 % TCA, dissolved in 150 μ l Tris base, and then counted by liquid scintillation.



Acknowledgements

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☐ 1: P43954. Reports Putative 4'-phosp...[gi:1175173]

BLINK, Conserved
Domains, Links

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 annotation updated: Sep 13, 2005.
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 Transferase.
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 ORGANISM Haemophilus influenzae
 Bacteria; Proteobacteria; Gammaproteobacteria; Pasteurellales;
 Pasteurellaceae; Haemophilus.
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 AUTHORS Fleischmann,R.D., Adams,M.D., White,O., Clayton,R.A.,
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 influenzae Rd
 JOURNAL Science 269 (5223), 496-512 (1995)
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